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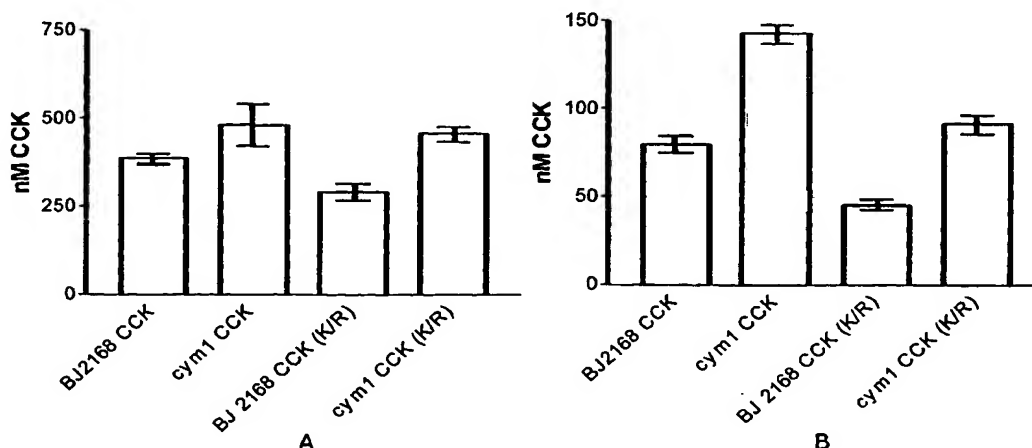
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(54) Title: METHODS FOR INCREASING THE PRODUCTION OF A RECOMBINANT POLYPEPTIDE FROM A HOST CELL



(57) Abstract: The present invention provides a host cell comprising a nucleic acid sequence encoding a recombinant polypeptide in which the production of a naturally occurring metalloprotease comprising a sequence provided in SEQ ID NO:1 has been reduced or inhibited by genetic manipulation. The present invention also relates to methods for enhancing the production of a polypeptide from a cell by disrupting the synthesis or activity of the metalloprotease. In particular, the present invention relates to methods for enhancing the secretion of recombinant polypeptides from host cells such as, but not limited to, yeast and bacterial cells. The metalloprotease is a member of the pitrilysin subfamily of proteases, characterized by comprising the sequence HXXEH (SEQ ID NO:1), where X is any amino acid.

## Methods for increasing the production of a recombinant polypeptide from a host cell

### Field of the Invention

The present invention relates to methods for enhancing the production of a polypeptide from a cell by disrupting the synthesis or activity of a metalloprotease from the clan ME (M16 family). In particular, the present invention relates to methods for enhancing the secretion of recombinant polypeptides from host cells such as, but not limited to, yeast and bacterial cells.

### Background of the Invention

Cholecystokinin (CCK) is a vertebrate neuroendocrine peptide hormone that is expressed in both gut and brain tissues. The maturation of bioactive CCK peptides depends on post-translational tyrosine sulfation, endoproteolytic cleavages, exoproteolytic trimmings and carboxyterminal amidation. The endoproteolytic processing of the N-terminus varies with CCK-83, -58, -39, -33, -22, -8 and -5 being identified. Most of the CCK peptides are synthesized after cleavage at a single Arg residue, however, CCK-22 requires processing after a single Lys residue.

Many recombinant polypeptides have been expressed in yeast as a fusion protein to the *Saccharomyces cerevisiae*  $\alpha$ -factor prepro-peptide to direct secretion through the secretory pathway. The best characterized yeast protease is the serine endoprotease, Kex2p (Fuller et al., 1989) which is involved in maturation of the  $\alpha$ -mating pheromone and of killer toxin (Julius et al., 1984). Another yeast protease is Yps1p belonging to the yapsin family of glycosyl-phosphatidylinositol (GPI)-anchored aspartyl proteases, which is able to rescue mating deficiency when overexpressed in a *kex2* mutant (Egel-Mitani et al., 1990). Expression of foreign proteins have shown that Yps1p and Yps2p contain endoprotease activity.

The use of host cells for the expression of recombinant polypeptides has greatly simplified the production of large quantities of commercially valuable polypeptides, which otherwise are obtainable only by purification from their native sources. There is a varied selection of expression systems currently available from which to choose for the production of any given polypeptide, including eubacterial and eukaryotic hosts. One important factor in the selection of an appropriate expression system is the ability of the host cell to produce adequate yields of the polypeptide. However, a problem frequently encountered is the high level of proteolytic enzymes produced by a given

host cell or in the culture medium. Accordingly, there is a need for further methods which enhance the production of a recombinant polypeptide from a host cell.

5 Metalloproteases are the most diverse of the four main types of protease, with more than 30 families identified to date. In these enzymes, a divalent cation, usually zinc, activates the water molecule. The zinc metalloproteases can be divided based on the zinc binding site into for example Zincins and Inverzincins (Hooper, N.M. 1994). The metal ion is held in place by amino acid ligands, usually three in number. The known metal ligands are His, Glu, Asp or Lys and at least one other residue which may play an  
10 electrophilic role is required for catalysis,. Of the known metalloproteases, around half contain an HEXXH motif, which has been shown in crystallographic studies to form part of the metal-binding site.

A number of proteases dependent on divalent cations for their activity have been  
15 shown to belong to a single family, peptidase M16. Included are insulinase, mitochondrial processing protease, pitrilysin, nardilysin and a number of bacterial proteins. These proteins do not share many regions of sequence similarity; the most noticeable is in the N-terminal section. This region includes a conserved histidine followed, two residues later, by a glutamate and another histidine. In pitrilysin, it has  
20 been shown that this HXXEH motif is involved in enzymatic activity (Becker et al. 1992); the two histidines bind zinc and the glutamate is necessary for catalytic activity. The X can be any amino acid. Non active members of this family have lost from one to three of these active site residues.

25 It has previously been suggested that one could provide host cells and methods of producing proteins by expressing significantly reduced levels of a genetical modification in order to express significantly reduced levels of a metalloprotease containing an HEXXH motif in a filamentous fungal host cell, in e.g. US 5,861,280 (WO 98/12300).

30 Others have provided a protease deficient filamentous fungus which is characterised in that the filamentous fungus contains a site selected disruption of DNA that results in the filamentous fungus having reduced metalloprotease activity and isolated DNA sequences encoding a protein having metalloprotease activity, which is obtainable from a filamentous fungus (WO 97/46689). Again this metalloprotease contains an HEXXH  
35 motif.

However, metalloproteases which can be reduced by a genetical modification in order to express significantly reduced levels of said metalloprotease in a non-filamentous

fungal host cell and other cells containing an motif other than HEXXH have never been described.

### Summary of the Invention

Whilst investigating the role various proteases play in processing proCCK in recombinant yeast, the present inventors surprisingly noted that deletion/disruption of *CYM1* enhanced recombinant polypeptide production and secretion. Furthermore, the present inventors have found that Cym1p belongs to a family of metalloproteases, the activity of which can be down-regulated to enhance the levels of recombinant polypeptide produced from a host cell.

Accordingly, in a first aspect the present invention provides a host cell comprising a nucleic acid sequence encoding a recombinant polypeptide in which the production of a naturally occurring metalloprotease comprising a sequence provided in SEQ ID NO:1 has been reduced or inhibited by genetic manipulation.

The host cell can be any cell which, in its native state, possesses the metalloprotease. Accordingly, the host cell can be a eukaryotic or prokaryotic cell. Examples of preferred eukaryotic cells include, but are not limited to, mammalian cells, plants cells and fungal cells. In a preferred embodiment, the host cell is a yeast cell. More preferably, the yeast cell is selected from, but not limited to, the group consisting of: *Saccharomyces* sp. such as *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, *Saccharomyces bayanus*, *Saccharomyces castellii* and *Saccharomyces kluyveri*, *Schizosaccharomyces* sp. such as *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida utilis*, *Candida cacaui*, and *Geotrichum fermentans*.

Metalloproteases are among the hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule. This is a characteristic shared with aspartic proteases, but in the metalloproteases a divalent metal cation, usually zinc, but sometimes cobalt or manganese, activates the water molecule. The metal ion is held in place by amino acid ligands usually 3 in number, the known metal ligands in metalloproteases are His, Glu, Asp or Lys residues.

Metalloproteases can be divided into two broad groups depending on the metal ions required for catalysis, and in the literature metalloproteases have been allocated into at least 8 different clans: MA, MB, MC, MD, ME, MF, MG and MH. Thus, illustrating the



complex diversity of this group of proteases. The allocation is based on different consensus sequences due to the ligand binding, and thus each family have different biological substrates and/or functions.

5 The metalloproteases which are to be down regulated according to the present invention is a member of the pitrilysin subfamily (ME) of proteases, characterized by comprising the sequence HXXEH (SEQ ID NO:1) where X is any amino acid. Presently more than 180 members are annotated in Swissprot to the ME clan. Thus, the most preferred embodiment, the metalloprotease comprises a consensus sequence provided  
10 in SEQ ID NO:1. In another preferred embodiment, the metalloprotease comprises a consensus sequence provided in SEQ ID NO:2. Even more preferably, the metalloprotease comprises a consensus sequence provided in SEQ ID NO:3. In addition, it is preferred that the metalloprotease comprises SEQ ID NO:1 and a glutamic acid residue between 70 and 80 amino acids C-terminal of the second His  
15 residue. Further, it is preferred that the metalloprotease comprises a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 as well as a sequence selected from the group of:

- i) any one of group consisting of SEQ ID NO's 4 to 15, and
- 20 ii) a sequence which is at least 80% identical to any one of SEQ ID NO's 4 to 15.

More preferably, the metalloprotease comprises SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 as well as a sequence selected from the group of:

- 25 i) any one of SEQ ID NO's 4 or 5, and
- ii) a sequence which is at least 80% identical to any one of SEQ ID NO's 4 or 5.

Preferably, the metalloprotease comprises a sequence which is at least 85% identical, such as at least 90% identical, such as at least 95% identical, and such as at least 99% identical to any one of SEQ ID NO's 4 to 15.  
30

In a particularly preferred embodiment, the metalloprotease comprises a sequence as provided in SEQ ID NO:4, or a sequence at least 80% identical, such as at least 90%,  
35 such as at least 95% and such as 99% identical, thereto.

The host cell can be genetically manipulated by any means known in the art as long as the production of the metalloprotease is reduced or inhibited when compared to a

parental host cell which has not been genetically manipulated. Such means of genetically manipulating the host cell include, but are not limited to; gene knockout, gene disruption, random or site directed mutagenesis, introduction of dominant-negative metalloproteases, RNA interference (RNAi) using dsRNA, catalytic nucleic acids (such as ribozymes and DNAzymes), and antisense nucleic acids. Preferably, the genetic manipulation acts directly upon the gene encoding the metalloprotease, the mRNA transcribed from the gene, or produces a protein that alters the activity of the metalloprotease such as a dominant negative mutant which competes with the metalloprotease for binding to a substrate but does not, for example, possess catalytic activity. However, the host cell may be genetically manipulated such that it indirectly affects the production or activity of the metalloprotease. For instance, the genetic manipulation can target a transcription factor involved in transcribing the mRNA encoded by the metalloprotease gene, thus at least reducing the levels of metalloprotease produced by the manipulated host cell.

Furthermore, the host cell may be further genetically manipulated such that it lacks at least one other naturally occurring protease of the host cell or has reduced activity for at least one other naturally occurring protease of the host cell. The protease can be any enzyme of which the inhibition increases the production of a recombinant polypeptide produced by the host cell. The protease can either be an endopeptidase, an aminopeptidase or a carboxypeptidase. Preferred proteases include serine proteases, aspartyl proteases, cysteine proteases and other metalloproteases.

In one embodiment, the host cell is a yeast cell and the other naturally occurring protease(s) is at least one protease encoded by any of the protease genes selected from the group consisting of; KEX2, YPS1 (previously known as YAP3), YPS2 (previously known as MKC7), YPS3, YPS6, YPS7, BAR1, STE13, KEX1, PRC1, PEP4 (also known as PRA1), APE1, APE2, APE3 and CPS1. Preferably, the host cell is a yeast cell and KEX2 production has been disrupted. Similar naturally occurring protease(s) within other host cells than yeast in addition to the metalloprotease specifically described here in could also be disrupted and/or genetically manipulated for an further additive enhancement.

The recombinant polypeptide can be any desired polypeptide which is capable of being produced in the host cell. The recombinant polypeptide can comprise a naturally occurring sequence or has been produced by the intervention of man (e.g. a mutant or truncation of a naturally occurring protein, or a fusion between at least two different

polypeptides). Typically, the recombinant polypeptide will be of commercial value, for example in the treatment of diseases.

5 The recombinant polypeptide can be any size. Typically, the recombinant polypeptide will range in size from about 30 amino acids to about 4,500 amino acids. In one embodiment, the recombinant polypeptide is between about 30 to about 200 amino acids in length.

10 In at least some host cell expression systems for producing recombinant polypeptides, it is desirable to direct the recombinant polypeptide to be secreted from the host cell. Thus, in a preferred embodiment, the nucleic acid comprises a sequence which encodes a signal for directing the recombinant polypeptide to be secreted from the host cell. Preferably, the signal is an N-terminal hydrophobic signal sequence. Such N-terminal hydrophobic signal sequences are known in the art, and include, for example but not  
15 limited to, the leader sequence originating from the fungal amyloglucosidase (AG) gene such as galA - both 18 and 24 amino acid versions e.g. from *Aspergillus sp.*, the  $\alpha$ -factor gene such as yeasts e.g. from *Saccharomyces sp.* and *Kluyveromyces sp.*, the P-factor of *Schizosaccharomyces sp.*, and the  $\alpha$ -amylase gene from *Bacillus sp.* In one embodiment, the recombinant polypeptide is expressed as a fusion of an N-terminal  
20 hydrophobic signal sequence and a second polypeptide sequence encoding the recombinant polypeptide which is from a different source than the signal sequence.

The nucleic acid encoding the recombinant polypeptide can be provided to the host cell using any technique known in the art. In one embodiment, the nucleic acid is inserted  
25 into the genome of the host cell using, for example, homologous recombination based techniques. In another embodiment, the nucleic acid is transfected or transformed into the host cell in an expression vector which remains extrachromosomal. For example, the expression vector can be a plasmid or a virus. Further, it is preferred that the vector comprises a selectable marker which can be used to selectively propagate host  
30 cells comprising the vector. Such selectable markers and the use thereof are also known in the art.

In a second aspect, the present invention provides a method of producing a recombinant polypeptide, the method comprising culturing a host cell according to the  
35 second aspect under suitable conditions such that the recombinant polypeptide is produced, and recovering the recombinant polypeptide.

Since the proteolytic action arising from the metalloprotease has been reduced or inhibited, the method of the second aspect of the invention improves the stability of the recombinant polypeptide produced by the host cell.

- 5 In a preferred embodiment, the recombinant polypeptide is secreted from the host cell. Furthermore, it is preferred that the secreted protein is recovered during exponential growth of a culture comprising the host cell.

- 10 Preferably, the quantity of the recovered recombinant polypeptide is higher than if a parental host cell was used. More preferably, the quantity of the recovered recombinant polypeptide is at 50% higher than if a parental host cell was used.

- In a third aspect, the present invention provides a method of cleaving a polypeptide at a basic residue, the method comprising contacting the polypeptide, in the presence of a  
15 divalent cation, with a metalloprotease comprising a sequence selected from the group of:

- i) any one of SEQ ID NO's 4 to 15, and
- ii) a sequence which is at least 80% identical to any one of SEQ ID NO's 4 to  
15.

20

In a particularly preferred embodiment, the metalloprotease comprises a sequence as provided in SEQ ID NO:4, or a sequence at least 80% identical, such as at least 90%, such as at least 95% and such as 99% identical, thereto.

- 25 Preferably, the metalloprotease cleaves the polypeptide at the C-terminal side of an amino acid, or sequence of amino acids, selected from the group consisting of; Lys, Arg, ArgArg, LysLys, ArgLys and LysArg. Accordingly, it is preferred that the polypeptide comprises Lys, Arg, ArgArg, LysLys, ArgLys or LysArg. Other sequence requirements may also be necessary for cleavage, however, these can readily be  
30 determined by routine experimentation.

Preferably, the divalent cation is selected from the group consisting of:  $Zn^{2+}$ ,  $Co^{2+}$  and  $Mn^{2+}$ .

- 35 The method of the third aspect can be performed *in vivo*, within a recombinant host cell producing the metalloprotease, or *in vitro* in suitable reaction conditions. Considering the present disclosure, the skilled addressee could readily perform the method of the third aspect. An example of an *in vitro* system for cleaving a polypeptide with the

defined metalloprotease is provided herein. In this instance, the polypeptide is contacted with the metalloprotease provided in a crude yeast cell extract in 0.1 M  $\text{NaH}_2\text{PO}_4$  (pH 4.5) and in the presence of 1 mM  $\text{Mn}^{2+}$  and 1 mM bestatin. In another example, the metalloprotease can be recombinantly produced as a fusion protein with a suitable "tag", such as a His-tag, which enables easy purification of the fusion protein. Preferably, such a "tag" is removed (for example by enzymatic cleavage) before the metalloprotease is exposed to the substrate polypeptide.

In a fourth aspect, the present invention provides a method of identifying an agent that inhibits the activity of a metalloprotease comprising a sequence provided in SEQ ID NO:1, the method comprising the steps of:

- a) incubating the metalloprotease with the agent, in the presence of a divalent cation and a suitable substrate;
- b) determining the activity of the metalloprotease on the substrate;
- c) comparing the activity obtained in step b) with the activity of a control sample that has not been incubated with the agent; and
- d) selecting an agent that inhibits the activity of the metalloprotease.

The substrate can be any polypeptide that can be cleaved by the metalloprotease and the cleavage event detected. One example disclosed herein is the use of CCK as a substrate, where the cleavage event is detected by the production of CCK-22. Similar assays can readily be developed for other substrates.

In a preferred embodiment of the fourth aspect, the metalloprotease comprises a sequence as provided in SEQ ID NO:4, or a sequence at least 80% identical, such as at least 90%, such as at least 95% and such as 99% identical, thereto.

In a fifth aspect, the present invention provides a method of producing a recombinant polypeptide, the method comprising culturing a host cell comprising a nucleic acid sequence encoding a recombinant polypeptide under suitable conditions such that the recombinant polypeptide is produced, and recovering the recombinant polypeptide wherein said culturing comprises the presence of an inhibitor of a metalloprotease comprising a sequence provided in SEQ ID NO:1.

Preferably, the inhibitor is identified according to a method of the fourth aspect.

As will be apparent, preferred features and characteristics of one aspect of the invention may be applicable to other aspects of the invention.

- 5 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.
- 10 The invention will hereinafter be described by way of the following non-limiting Figures and Examples.

### Brief Description of the Accompanying Drawings

**Figure 1.** Cholecystokinin expression construct. PreproMf $\alpha$ 1p-proCCK fusion protein  
15 with the amino acid sequences around the fusion site and of the primary cleavage sites shown. The major forms of secreted CCK with their N- and C-terminal amino acid residues are shown below.

**Figure 2.** CCK-22 maturation in cells and media as a function of cell growth. BJ2168  
20 expressing preproMf $\alpha$ 1p-proCCK fusion protein. The CCK-22 immuno-reactivity was measured by RIA using Ab 89009 and total CCK content measured with Ab 89009 after tryptic cleavage. Open circles represent the fraction of secreted CCK-22, whereas the intracellular fraction of CCK-22 is presented as filled triangles. The cell growth was measured by OD<sub>600</sub> (open squares). The data represent mean of two independent  
25 experiments.

**Figure 3.** Chromatographic analyses of normal and K $\rightarrow$ A mutated CCK secreted from BJ2168. Media from yeast transformed with pRS426 preproMf $\alpha$ 1p-proCCK and pRS426 preproMf $\alpha$ 1p-proCCK (K $\rightarrow$ A) were subjected to G-50 gel chromatography and the CCK  
30 immuno-reactivity was measured with Ab 7270 specific for Gly extended CCK (A and C) and Ab 89009, which is specific for the N-terminus of CCK-22 (B and D).

**Figure 4.** *In vitro* protease assay including inhibitors and activators. The fraction of CCK-22 was calculated from the immuno-reactivity using Ab 89009 divided by the total  
35 amount of mature and N-terminal extended CCK-22 measured with Ab 89009 after trypsin treatment. A, Effect of different inhibitors. B, Protease reactivation by addition

of 1.2 mM divalent metal ions to extracts where the activity had been inhibited with 1 mM EDTA. The data represent mean  $\pm$  SD of three independent experiments.

**Figure 5.** Protease reactivation by  $Zn^{2+}$  and  $Mn^{2+}$ . *In vitro* protease assays performed with cell extracts from LJY123, where the activity was inhibited with 1 mM EDTA (filled squares) and reactivated by addition of 1.2 mM  $Mn^{2+}$  (open circles) or 1.2 mM  $Zn^{2+}$  (filled circles). The activity was measured as the fraction of matured CCK-22 after 30, 60 and 120 min incubation. The fraction of CCK-22 was calculated from the immuno-reactivity using Ab 89009 divided by the total amount of mature and N-terminal extended CCK-22 measured with Ab 89009 after trypsin treatment. The data are represented by mean  $\pm$  SD (n=3).

**Figure 6.** Extracellular CCK-22 maturation by members of the yapsin family. The ability of intact cells to process extracellular CCK was analysed as described under "Experimental Procedures" for BY4705 and the isogenic *yps1*, *yps1 yps3* and *yps1 yps2 yps3* strains. The fraction of CCK-22 was calculated from the immuno-reactivity using Ab 89009 divided by the total amount of total CCK measured with Ab 89009 after trypsin treatment. The data are represented by mean  $\pm$  SD (n=4). Statistics were performed using unpaired t test as described in experimental procedures (\*\*\* = P<0.001, \*\* = P<0.01 and \* = P<0.05).

**Figure 7.** Increased proteolysis following *Cym1p* overexpression. *In vitro* protease assays performed with cell extracts from BJ2168 transformed with an empty pRS425 plasmid (A) and with pRS425 containing *CYM1* (B). The CCK-22 immuno-reactivity was measured over time using Ab 89009 (filled squares and circles) and the total amount of mature and N-terminal extended CCK-22 measured with Ab 89009 after trypsin treatment (open squares and circles). The data represent mean  $\pm$  SD of three independent experiments.

**Figure 8.** Effects of *KEX2* and *CYM1* deletions on proCCK secretion and CCK-22 maturation. Yeast cells transformed with the proCCK expression construct were harvested during exponential phase and the media collected. The intracellular (A) and extracellular (B) amount of total CCK was measured with Ab 89009 after trypsin treatment. The fraction of intracellular (C) and secreted (D) CCK-22 was calculated as the immuno-reactivity measured with Ab 89009 before tryptic cleavage divided with the total amount of CCK measured in (A) and (B). The *kex2*, *cym1* and *kex2 cym1* strains are isogenic to BJ2168. The data are given as mean  $\pm$  SD (n=4). Statistics were

performed using unpaired t test (\*\*\* =  $P < 0.001$ , \*\* =  $P < 0.01$  and \* =  $P < 0.05$ ). The stars enclosed in brackets are a comparison between the *kex2* and *kex2 cym1* strain.

**Figure 9.** Intracellular degradation of CCK depends on Cym1p cleavage to CCK-22.

- 5 Expression of wild type CCK, preproMf $\alpha$ 1p-proCCK, and the CCK mutant, preproMf $\alpha$ 1p-proCCK (K $\rightarrow$ A) in BJ2168 and a *CYM1* disrupted strain isogenic to BJ2168. The cells were sedimented during exponential growth and the total amount of CCK (hatched bars) was measured after trypsin and carboxypeptidase B treatment with Ab 7270 specific for Gly-extended CCK. The amount of mature Gly-extended (white bars), which  
10 is dependent on translocation into the secretory pathway, Kex2p and carboxypeptidase activity is measured as the immuno-reactivity using Ab 7270 before tryptic cleavage and carboxypeptidase B treatment. The data are given as mean  $\pm$  SD (n=3).

**Figure 10.** Aspartyl proteases involved in the maturation of CCK-22. Expression of wild  
15 type proCCK in BY4705 and the isogenic yapsin deletion strains of *YPS1*, *YPS2* and *YPS3*. The intra- (A) and extracellular (B) fraction of synthesised CCK-22 was measured during exponential growth. The fraction of mature CCK-22 was calculated as the immuno-reactivity measured with Ab 89009 before tryptic cleavage divided with the total amount of CCK. The data are represented by mean  $\pm$  SD (n=3). Statistics were  
20 performed using unpaired t test as described in experimental procedures (\*\*\* =  $P < 0.001$ , \*\* =  $P < 0.01$  and \* =  $P < 0.05$ ).

**Figure 11.** Cym1p processing C-terminally to both Lys and Arg residues. *CYM1*  
deletion enhance the amount of secreted CCK more than two fold of both wild type CCK  
25 and the Lys<sup>61</sup>  $\rightarrow$  Arg<sup>61</sup> mutant. Expression of wild type CCK, preproMf $\alpha$ 1p-proCCK, and the CCK mutant, preproMf $\alpha$ 1p-proCCK (Lys<sup>61</sup>  $\rightarrow$  Arg<sup>61</sup>) in BJ2168 and a *CYM1* disruptant isogenic to BJ2168. Yeast cells were harvested during exponential phase and the media collected. The intracellular (A) and extracellular (B) amount of total CCK was measured with Ab 89009 after trypsin treatment. The data are given as mean  $\pm$  SD (n=3).  
30

**Figure 12.** Secreted proCCK fragments identified by mass spectrometry. The CCK-  
numbers refer to C-terminal amidated CCK. The molecular masses are given as  
monoisotopic values except for \* which denote average value. Strain A, vacuolar  
protease-deficient strain (BJ2168), and B, the isogenic strain with *KEX1 KEX2*  
35 disruptions (LJY22).



**Figure 13.** Model for the production of the C-terminally extended CCK (A) and GLP2 (B). Expression of these fusion peptides should be performed in a *sec61* mutant, or the pre-sequence of the  $\alpha$ -mating factor should be removed to avoid translocation into the ER. The amino acid sequences around the fusion sites are shown. Underlined are the N- and C-terminal amino acids of the Gly-extended CCK-22 and GLP1.

#### Figure 14

A. The preproMf $\alpha$ 1p-proBNP expression construct.

B. The preproMf $\alpha$ 1p-KREAEA-BNP-32 expression construct.

C. The preproMf $\alpha$ 1p-KR-BNP-32 expression construct.

#### Figure 15

A. The preproMf $\alpha$ 1p- proBNP expression construct transformed in BJ2168, LJY430 (*cym1* mutant), LJY431 (*yps1* mutant) and LJY432 (*cym1 yps1* double mutant). Media was analysed from cells that have reached stationary phase using Ab 98192 that is specific for the N-terminus of proBNP. The *cym1*, *yps1*, and *cym1yps1* strains are isogenic to BJ2168. The data are given as mean  $\pm$  SD (n=3). Statistics were performed using unpaired t test as described in experimental procedures (\*\*\* = P<0.001, \*\* = P<0.01 and \* = P<0.05). The stars enclosed in brackets are comparisons are between the wild type strain, BJ2168 vs. *cym1*, BJ2168 vs. *yps1* and *yps1* vs. *yps1cym1*.

(ns, = not significant).

B. Analysis of proCCK fragments secreted from a *cym1* mutant. Media containing 10 pmole proBNP was applied to Superdex 200 column on a Akta purifier system. The proBNP content in the collected fractions were measured using Ab. 98192, that is specific for the N-terminus of proBNP.

#### Key to the Sequence Listing

SEQ ID NO:1 – Consensus sequence for pitrilysin proteases.

SEQ ID NO:2 – Consensus sequence for at least some pitrilysin proteases.

SEQ ID NO:3 – Consensus sequence for at least some pitrilysin proteases.

SEQ ID NO:4 – *Saccharomyces cerevisiae* Cym1p (Swissprot Accession No. P32898).

SEQ ID NO:5 - *Schizosaccharomyces pombe* C119.7 (Swissprot Accession No. O42908).

SEQ ID NO:6 - *Clostridium perfringens* HypA protein (Swissprot Accession No. Q46205).

5 SEQ ID NO:7 - *Borrelia burgdorferi* protein BB0228 (Swissprot Accession No. O51246).

SEQ ID NO:8 - *Caenorhabditis elegans* C05D11.1 protein (Swissprot Accession No. P48053).

SEQ ID NO:9 - *E. coli* protease III (Swissprot Accession No. P05458).

SEQ ID NO:10 - Rat NRD convertase (Swissprot Accession No. P47245).

10 SEQ ID NO:11 - Human insulysin (Swissprot Accession No. P14735).

SEQ ID NO:12 - *Arabidopsis thaliana* CPE (Genbank Accession No. T03302).

SEQ ID NO:13 - Human metalloprotease I (GenBank Accession No. AAH01150) in part, the full sequence (Swissprot Accession No. O95204).

SEQ ID NO:14 - *Bacillus subtilis* zinc protease ymxG (GenBank Accession No. Q04805).

15 SEQ ID NO:15 - *Mycobacterium tuberculosis* zinc protease Rv2782c (GenBank Accession No. O33324).

SEQ ID NO's 16 to 42 - Oligonucleotides.

SEQ ID NO's 43 to 52 - Sequences provided in Figure 12.

SEQ ID NO's 53 to 55 - Sequences provided in Figure 1.

20 SEQ ID NO's 56 to 65 - Oligonucleotides.

SEQ ID NO:66 - Consensus sequence for at least some pitrilysin proteases.

SEQ ID NO:67 - Consensus sequence for at least some pitrilysin proteases.

SEQ ID NO:68 - Consensus sequence for at least some pitrilysin proteases.

## 25 Detailed Description of the Invention

The present invention provides a host cell useful for the expression of a polypeptide, said cell being genetically manipulated in order to at least produce reduced levels of a defined metalloprotease, when compared to the parental cell. The host cell will thus be able to express a protein of interest in higher quantity due to the proteolytic action of the metalloprotease has been reduced or inhibited which improves the stability of the protein of interest.

By the method of the invention, the proteolytic action of the metalloprotease has been reduced or inhibited, thereby improving the stability of the product obtained.

35

Thus, one embodiment of the present invention relates to a host cell useful for the expression of a protein of interest, wherein said cell has been genetically modified in

order to express significantly reduced levels of a metalloprotease comprising a HXXEH motif (SEQ ID NO 1) compared to the corresponding non-modified cell when cultured under identical conditions.

- 5 The metalloproteases which are to be down regulated according to the present invention do not share many regions of sequence similarity; the most noticeable is in the N-terminal section. This region includes a conserved histidine followed two residues later by a glutamate and another histidine. In pitrilysin, it has been shown that this HXXEH motif is involved in enzymatic activity; the two histidines bind zinc and the glutamate is necessary for catalytic activity. Non active members of this family have lost from one to three of these active site residues.

- The metalloprotease family which are to be down regulated according to the present invention is presently classified as member of clan ME, family M16. This family is currently divided into 4 subfamilies:

M16A comprising pitrilysin

M16B comprising mitochondrial processing peptidase beta-subunit (*Saccharomyces cerevisiae*)

- 20 M16C comprising eupitrilysin (*Homo sapiens*)

M44 comprising vaccinia virus-type metalloindopeptidase (vaccinia virus).

- Sequence alignments of these proteins show several sequence similarities. These sequence similarities are highly conserved and can be used to distinguish members of this family from non-members.

Among such sequence similarities several individual amino acids are highly conserved and are easily recognisable in specific positions navigated from the HXXEH motif.

- 30 Thus, one embodiment of the present invention relates to a host cell, wherein the metalloprotease comprises a glutamic acid residue between 70 and 80 amino acids C-terminal of the second His residue in the HXXEH motif.

- A further embodiment of the present invention relates to a host cell, wherein the metalloprotease comprises a glycine residue 3 amino acids N-terminal of the first His residue in the HXXEH motif.

Another embodiment of the present invention relates to a host cell, wherein the metalloprotease comprises a glycine residue 5 amino acids C-terminal of the second His residue in the HXXEH motif.

- 5 One further embodiment of the present invention relates to a host cell wherein the metalloprotease comprises a lysine residue 8 amino acids C-terminal of the second His residue in the HXXEH motif.

- Also, one embodiment of the present invention relates to a host cell, wherein the  
10 metalloprotease comprises a tyrosine residue 9 amino acids C-terminal of the second His residue in the HXXEH motif.

- Furthermore, the present invention relates to a host cell, wherein the metalloprotease comprises a proline residue 10 amino acids C-terminal of the second His residue in the  
15 HXXEH motif.

- Among the sequence similarities several regions of amino acids are also highly conserved and are easily recognised. Thus, in a presently preferred embodiment the invention relates to a host cell wherein the metalloprotease comprises the consensus  
20 sequence SEQ ID NO 2.

In another presently preferred embodiment, the invention relates to a host cell, wherein the metalloprotease comprises the consensus sequence SEQ ID NO 3.

- 25 In a presently most preferred embodiment, the invention relates to a host cell, wherein the metalloprotease comprises a NAXTXXXXT motif between 20 and 30 amino acids C-terminal of the second His residue in the HXXEH motif.

- In a presently another preferred embodiment, the invention relates to a host cell,  
30 wherein the metalloprotease comprises the consensus sequence SEQ ID NO 66-68. One embodiment of the present invention relates to a host cell useful for the expression of a protein of interest, wherein said cell has been genetically modified in order to express significantly reduced levels of a metalloprotease which is at least 80% identical to the any of SEQ ID NO: 4-15, as compared to a parental cell.

35

In the present context, the term "protein of interest" relates to any of the numerous naturally native occurring extremely complex substances such as but not limited to proteins, enzymes and/or antibodies that consist of amino acid residues joined by

peptide bonds. It is an object of preferred embodiments of the present invention to provide such native proteins which are products of the host cell itself and/or heterologous proteins, fusion proteins, recombinant proteins, eukaryotic proteins, prokaryotic proteins, lysosomal proteins, vacuolar proteins, precursor proteins, zymogene proteins, prepro-proteins, and secreted proteins.

Preferred embodiments of the claimed method are advantageous due to the higher production of the protein of interest, thus any increase of the amount of the protein of interest when produced in a host cell modified as described herein compared to the amount produced in the corresponding non-modified cell when cultured under identical conditions are within the scope of the present invention.

One assay in which a skilled addressee could evaluate enhanced production of the protein of interest in a host cell modified as described here in and compared to the amount produced in the corresponding non-modified cell, is by culturing the two different host cells under identical condition, and measure the amount of produced protein of interest by radio-immune assay using an antibody specific for the protein of interest. One such assay is describe in more detail in the examples of the present description.

One embodiment of the present invention relates to a host cell, wherein the total amount of the protein of interest is increased at least 5% compared to the corresponding non-modified cell when cultured under identical conditions, such as at least 10% compared to the corresponding non-modified cell when cultured under identical conditions, such as at least 20% compared to the corresponding non-modified cell when cultured under identical conditions, such as at least 50% compared to the corresponding non-modified cell when cultured under identical conditions, such as at least 100% compared to the corresponding non-modified cell when cultured under identical conditions, such as at least 200% compared to the corresponding non-modified cell when cultured under identical conditions, or even at least 1000% or compared to the corresponding non-modified cell when cultured under identical conditions.

In the present context, the term "host cell" relates to any cell capable of producing the protein of interest. Thus, in one preferred embodiment, the host is a prokaryotic cell. In another preferred embodiment, the host cell is a eukaryotic cell, such as but not limited to a filamentous fungal cell and a non-filamentous fungal cell. Non limiting examples hereof are a strain of *Saccharomyces*, especially *Saccharomyces cerevisiae*.

All the features described herein relating to the methods of the present invention are also applicable as embodiments relating to the host cells, and vice versa.

- 5 The method described in the present application relates to the production of a protein of interest in a host cell, wherein said host cell has been genetically modified in order to express significantly reduced levels of a metalloprotease which is at least 80% identical to the SEQ ID NO: 4 as compared to a parental cell, when cultured under identical conditions, comprising
- 10 a) introducing into the host cell a nucleic acid sequence encoding the protein of interest;
- b) cultivating the host cell of step (a) in a suitable growth medium for
- 15 production of the protein of interest and
- c) isolating the protein of interest.

One embodiment of the present invention relates to a method for the production of a

20 protein of interest in a host cell, wherein the host cell has been genetically modified by a method selected from the group comprising gene knock-out, gene disruption, random or site directed mutagenesis, introduction of dominant-negative metalloproteases, RNA interference (RNAi) using dsRNA, catalytic nucleic acids (such as ribozymes and DNazymes), antisense nucleic acids or a combination thereof.

25 In a presently most preferred embodiment, the host cell is essentially free of any metalloprotease activity.

One preferred embodiment of the present invention relates to a method for the

30 production of a protein of interest in a host cell, in which the protein of interest is a eukaryotic protein, selected from the group comprising insulin, growth hormone, glucagon, somatostatin, interferon, adrenocorticotrophic hormones, angiotensinogen, atrial natriuretic peptides, dynorphin, endorphines, galanin, gastrin, gastrin releasing peptides, neuropeptide Y fragments, pancreastatin, pancreatic polypeptides, secretin,

35 vasoactive intestinal peptide, growth hormone releasing factor, melanocyte stimulating hormone, neurotensin, adrenal peptide, parathyroid hormone and related peptides, somatostatin and related peptides, brain natriuretic peptide, calcitonin, corticotropin releasing factor (CRF), cocaine amphetamine regulated transcript (CART), thymosin,

urotensin, glucagon and glucagon like peptides (GLP-1 and GLP-2), somatostatin, interferon, a vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), factor VII, factor VIII, factor V, factor IX, interleukins, urokinase, erythropoietin (EPO), chymosin, tissue plasminogen activator, Positive cofactor 2  
5 glutamine/Q-rich-associated protein (PCAP), peptide tyrosine tyrosine (PYY), ghrelin, orexin, Beta-neoendorphin-dynorphin precursor, CCK or serum albumin.

Another preferred embodiment of the present invention relates to a method for the production of a protein of interest in a host cell, in which the protein of interest is a  
10 protein of fungal origin, selected from the group comprising an amylolytic enzyme, an alpha-amylase, a beta-amylase, a glyco-amylase, a alpha-galactosidase, a cellulytic enzyme, a lipolytic enzyme, a xylanolytic enzyme, a proteolytic enzyme, an oxidoreductase, a peroxidase, a laccase, a pectinase, or a cutinase.

15 A further preferred embodiment of the present invention relates to a method for the production of a protein of interest in a host cell, in which the protein of interest is a bacterial protein, selected from the group comprising an amylolytic enzyme, an alpha-amylase, a beta-amylase, a glyco-amylase, a beta-galactosidase, a cellulytic enzyme, a lipolytic enzyme, a xylanolytic enzyme, a proteolytic enzyme, an oxidoreductase, a  
20 peroxidase, a laccase, a pectinase, or a cutinase.

A special embodiment of the present invention relates to a method for production of a protein of interest in a host cell, in which the protein of interest is a precursor, i.e. a zymogen, a hybrid protein, a protein obtained as a pro sequence or pre-pro sequence,  
25 or in unmaturation form.

#### General Molecular Biology

Unless otherwise indicated, the recombinant DNA techniques utilised in the present  
30 invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical  
35 Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates

and Wiley-Interscience (1988, including all updates until present), *Methods in Enzymology*. Vol 194. *Guide to Yeast Genetics and Molecular Biology*. (1991) Ed Gunthrie and Fink Academic Press, *Methods in Microbiology* Vol. 26. *Yeast Gene Analysis*. (1998) Ed. Brown and Tuite. Academic Press, Miller, J. H. (1992) *A Short Course in Bacterial Genetics* (Manual, L., ed), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, Johnston, J. R (1994) *Molecular Genetics of Yeast* (A Practical Approach) Oxford University Press, Oxford., and *Molecular Genetics of Yeast: A Practical Approach*, Ed. J.R. Johnston, IRL Press (1994) and are incorporated herein by reference.

10

The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 15 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 15 amino acids. More preferably, the query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. Even more preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. More preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the query sequence is at least 500 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 500 amino acids.

#### Pitrilysin Subfamily of Metalloproteases

25

The pitrilysin subfamily of metalloproteases is characterized by the presence of a HXXEH (SEQ ID NO:1) motif. A general review of this subfamily is provided by Rawlings and Barrett (1995). Members of this family include, but are not limited to, *S. cerevisiae* Cym1p (SEQ ID NO:4) (Swissprot Accession No. P32898), *Schizosaccharomyces pombe* C119.7 (SEQ ID NO:5) (Swissprot Accession No. O42908), *Clostridium perfringens* HypA protein (SEQ ID NO:6) (Swissprot Accession No. Q46205), *Borrelia burgdorferi* protein BB0228 (SEQ ID NO:7) (Swissprot Accession No. O51246), *Caenorhabditis elegans* C05D11.1 protein (SEQ ID NO:8) (Swissprot Accession No. P48053), *E. coli* protease III (also known as pitrilysin) (SEQ ID NO:9) (Swissprot Accession No. P05458), rat NRD convertase (SEQ ID NO:10) (Swissprot Accession No. P47245), human insulysin (SEQ ID NO:11) (Swissprot Accession No. P14735), *Arabidopsis thaliana* CPE (SEQ ID NO:12) (Genbank Accession No. T03302), human



metalloprotease I (in part) (SEQ ID NO:13) (GenBank Accession No. AAH01150) (the full sequence: Swissprot Accession No. O95204), *Bacillus subtilis* zinc protease ymxG (SEQ ID NO:14) (GenBank Accession No. Q04805), and *Mycobacterium tuberculosis* zinc protease Rv2782c (SEQ ID NO:15) (GenBank Accession No. O33324). For *E. coli* protease III (SEQ ID NO:9) it has been shown that the His residues of SEQ ID NO:1, as well as Glu-169, are involved in divalent cation binding whilst the Glu residue flanked by the His residues is a catalytic residue.

A gene encoding a pitrilysin metalloprotease can readily be identified by screening by hybridization for nucleic acid sequences coding for all of, or part of, the metalloprotease, e.g. by using synthetic oligonucleotide probes, that may be prepared on the basis of a cDNA sequence, e.g. the nucleotide sequences encoding any one of the metalloproteases presented as SEQ ID NO's: 4 to 15, in accordance with standard techniques.

15

#### Genetic Manipulations

The host cell of the invention which is genetically manipulated in order to produce reduced levels of the defined metalloprotease may be modified using standard recombinant DNA technology known to the person skilled in the art. The gene sequence responsible for the production of the metalloprotease may be inactivated or eliminated entirely.

In a particular embodiment, the host cell of the invention is one genetically manipulated at the coding or regulatory regions of the metalloprotease gene. Known and useful techniques include, but are not limited to, gene knockout, gene disruption, random or site directed mutagenesis, introduction of dominant-negative metalloproteases, RNA interference (RNAi) using dsRNA, catalytic nucleic acids (such as ribozymes and DNAzymes), and antisense nucleic acids, or a combination thereof.

30

Mutagenesis may be performed using a suitable physical or chemical mutagenizing agent. Examples of a physical or chemical mutagenizing agent suitable for the present purpose includes ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulfite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable

35

conditions for the mutagenesis to take place, and selecting for mutated cells having a significantly reduced production of metalloprotease.

Genetic manipulation may also be accomplished by the introduction, substitution or  
5 removal of one or more nucleotides in the metalloprotease coding sequence or a  
regulatory element required for the transcription or translation thereof. Nucleotides  
may, for example, be inserted or removed so as to result in the introduction of a stop  
codon, the removal of the start codon or a change of the open reading frame. The  
modification or inactivation of the structural sequence or a regulatory element may be  
10 accomplished by site-directed mutagenesis or PCR generated mutagenesis in  
accordance with methods known in the art.

A convenient way to inactivate or reduce the metalloprotease production of a host cell  
is based on the principles of gene interruption. This method involves the use of a DNA  
15 sequence corresponding to the endogenous gene or gene fragment which it is desired  
to destroy. The DNA sequence is *in vitro* mutated to a defective gene and transformed  
into the host cell. By homologous recombination, the defective gene replaces the  
endogenous gene or gene fragment. It may be desirable that the defective gene or  
gene fragment encodes a marker which may be used for selection of transformants in  
20 which gene encoding the metalloprotease has been modified or destroyed.

The term "antisense" as used herein refers to nucleotide sequences which are  
complementary to a specific nucleic acid sequence. Antisense molecules may be  
produced by any method, including synthesis by ligating the gene(s) of interest in a  
25 reverse orientation to a viral promoter which permits the synthesis of a complementary  
strand. Once introduced into a host cell, this transcribed strand combines with natural  
sequences, in this instance that encoding the metalloprotease, produced by the cell to  
form duplexes. These duplexes then block either the further transcription or translation.  
In this manner, mutant phenotypes may be generated.

30 The term "catalytic nucleic acid" refers to a DNA molecule or DNA-containing molecule  
(also known in the art as a "deoxyribozyme") or an RNA or RNA-containing molecule  
(also known as a "ribozyme") which specifically recognizes a distinct substrate and  
catalyzes the chemical modification of this substrate. The nucleic acid bases in the  
35 catalytic nucleic acid can be bases A, C, G, T and U, as well as derivatives thereof.  
Derivatives of these bases are well known in the art.

Typically, the catalytic nucleic acid contains an antisense sequence for specific recognition of a target nucleic acid, and a nucleic acid cleaving enzymatic activity, also referred to herein as the "catalytic domain". The types of ribozymes that are particularly useful in this invention are the hammerhead ribozyme (Haseloff and Gerlach, 1988) and the hairpin ribozyme (Shippy et al., 1999).

Ribozymes useful for the methods of the invention, and DNA encoding the ribozymes, can be chemically synthesized using methods well known in the art. The ribozymes can also be prepared from a DNA molecule (that upon transcription yields an RNA molecule) operably linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with RNA polymerase and nucleotides. In a separate embodiment, the DNA can be inserted into an expression cassette or transcription cassette. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase. Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

dsRNA (RNAi) is particularly useful for specifically inhibiting the production of a particular protein. This technology relies on the presence of dsRNA molecules that contain a sequence that is essentially identical to the mRNA of the gene of interest, in this case a mRNA encoding the metalloprotease. Conveniently, the dsRNA is produced in a single open reading frame in a recombinant vector or host cell, where the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a loop structure. The design and production of suitable dsRNA molecules for genetic manipulation is well known within the capacity of a person skilled in the art, particularly considering Waterhouse et al. (1998), Elbashir et al. (2001), WO 99/32619, WO 99/53050, WO 99/49029, and WO 01/34815.

Owing to the genetic manipulation, the host cell of the invention expresses significantly reduced levels of the metalloprotease. In a preferred embodiment, the level of metalloprotease expressed by the host cell is reduced more than about 25%, such as more than about 30%, such as more than about 35%, such as more than about 40%, such as more than about 45%, such as more than about 50%, such as more than about 55%, such as more than about 60%, such as more than about 65%, such as more than about 70%, such as more than about 75%, such as more than about 80%,

such as more than about 85%, such as more than about 90%, such as more than about 95%, such as more than about 98%, and such as more than about 99%.

In a presently most preferred embodiment, the product expressed by the host cell is  
5 essentially free of any activity of the defined metalloprotease.

In the present context, the term "essentially free" relates to a host, wherein the metalloprotease expressed by said host cell is reduced to a level, where the function of said metalloprotease has no biologically significant reducing influence on the production  
10 of the protein of interest.

#### Protein of Interest

The terms "polypeptide", "protein" and "peptide" are used herein interchangeably and  
15 in the present context relates to any of the numerous naturally occurring extremely complex substances such as but not limited to enzymes or antibodies that consist of amino acid residues joined by peptide bonds, contain the elements carbon, hydrogen, nitrogen, oxygen, usually sulphur, and occasionally other elements such as but not limited to phosphorus or iron, that are essential constituents of all living cells, that are  
20 in nature synthesised from raw materials by plants but assimilated as separate amino acids by animals, that are both acidic and basic and usually colloidal in nature although many have been crystallised, and that are hydrolyzable by acids, alkalies, proteolytic enzymes, and putrefactive bacteria to polypeptides, to simpler peptides, and ultimately to alpha-amino acids.

25

As defined herein, a "recombinant polypeptide" is a protein which is not native to the host cell, or a native polypeptide in which modifications have been made to alter the native sequence, or a native protein whose expression is quantitatively altered as a result of a manipulation of a native regulatory sequence required for the expression of  
30 the native protein, such as a promoter, a ribosome binding site, etc., or other manipulation of the host cell by recombinant DNA techniques.

Owing to the absence or reduction in activity of the defined metalloprotease, at least a portion of the recombinant polypeptides expressed by the host cell may also be a  
35 precursor protein, i.e. a zymogen, a hybrid protein, a protein obtained as a pro sequence or pre-pro sequence, or in unmaturation form.

In a more specific embodiment, the recombinant polypeptide is of eukaryotic origin, such as insulin, adrenocorticotrophic hormones, angiotensinogen, atrial natriuretic peptides, dynorphin, endorphines, galanin, gastrin, gastrin releasing peptides, neuropeptide Y fragments, pancreastatin, pancreatic polypeptides, secretin, vasoactive  
5 intestinal peptide, growth hormone releasing factor, melanocyte stimulating hormone, neurotensin, adrenal peptide, parathyroid hormone and related peptides, somatostatin and related peptides, brain natriuretic peptide, calcitonin, corticotropin releasing factor (CRF), cocaine amphetamine regulated transcript (CART), thymosin, urotensin, glucagon and glucagon like peptides (GLP-1 and GLP-2), somatostatin, interferon, a  
10 vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), factor VII, factor VIII, factor V, factor IX, interleukins, urokinase, erythropoietin (EPO), chymosin, tissue plasminogen activator, CCK or serum albumin.

With specific regard to "glucagon and glucagon like peptides", this term as used herein  
15 may refer to polypeptides of human origin or from other animals and recombinant or semisynthetic sources and include all members of the glucagon family, such as GRPP (glicentine related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2), including truncated and/or N-terminally extended forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y,  
20 GLP-2 A19T+34Y. GLP2 A2G and GLP-2 A19T, and other analogues having from 1 to 3 amino acid changes, additions and/or deletions.

#### Host Cells and the Expression of Recombinant Polypeptides Therefrom

25 The host cells for use in the present invention can be prokaryotic or eukaryotic. The eukaryotic host cells for use in the present invention can be, for example, fungal, mammalian, plant or insect cells. Preferably, the host cells are yeast cells.

In order to produce the desired polypeptide, the host cell of the invention comprises a  
30 nucleic acid sequence encoding the recombinant polypeptide as well as regulatory sequences for directing the expression of the desired product such as regions comprising nucleotide sequences necessary or e.g. transcription, translation and termination. The genetic design of the host cell of the invention may be accomplished by the person skilled in the art, using standard recombinant DNA technology for the  
35 transformation or transfection of a host cell.

Preferably, the host cell is modified by methods known in the art for the introduction of an appropriate expression cassette in, for example a plasmid or a viral vector, comprising the nucleic acid encoding the recombinant polypeptide. The expression cassette may be introduced into the host cell by a number of techniques including, but not limited to, as an autonomously replicating plasmid or integrated into the chromosome.

Expression cassettes may contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules encoding the recombinant polypeptide. In particular, recombinant nucleic acid molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, arthropod and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, *oxy-pro*, *omp/lpp*, *rrnB*, bacteriophage lambda, bacteriophage T7, T7lac, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Transcription control sequences of the present invention are most preferably naturally occurring transcription control sequences associated with yeast. Suitable promoters for *S. cerevisiae* include the MF $\alpha$ 1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI1 and PGK1 promoters, TRP1 promoter, CYC1 promoter, CUP1 promoter, PHOS promoter, ADH1 promoter, and HSP promoter. A suitable promoter in the genus *Pichia* is the AOX1 (methanol utilisation) promoter.

Recombinant polypeptides of the present invention may also (a) contain secretory signals to enable an expressed polypeptide to be secreted from the cell that produces the polypeptide and/or (b) contain fusion sequences which lead to the expression of fusion proteins. Examples of suitable signal segments include any signal segment  
5 capable of directing the secretion of the fusion protein. Preferred signal segments include, but are not limited to, tissue plasminogen activator (t-PA), interferon, interleukin, growth hormone, histocompatibility and viral envelope glycoprotein signal segments, the leader sequence originating from the fungal amyloglucosidase (AG) gene such as galA - both 18 and 24 amino acid versions e.g. from *Aspergillus sp.*, the  $\alpha$ -  
10 factor gene of yeasts e.g. from *Saccharomyces sp.* and *Kluyveromyces sp.*, the P-factor of *Schizosaccharomyces sp.*, and the  $\alpha$ -amylase gene from *Bacillus sp.*, as well as natural signal sequences.

The cloning vehicle may also comprise a selectable marker, e.g. a gene, the product of  
15 which complements a defect in the host cell, or one which confers antibiotic resistance, such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable cloning  
20 vehicles containing the information necessary for replication, are well known to persons skilled in the art.

Recombinant DNA technologies can be used to improve the expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the  
25 nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules useful for the methods of the present invention include, but are not limited to, operably linking the  
30 nucleic acid molecule to high-copy number plasmids, integration of the nucleic acid molecule into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification  
35 of nucleic acid molecule to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts. The activity of an expressed recombinant polypeptide of the present invention may be improved by fragmenting, modifying, or derivatizing polynucleotide molecules encoding such a protein.

## Methods of Producing Recombinant Polypeptides

- Host cells that have been transfected or transformed with the nucleic acid encoding the
- 5 recombinant polypeptide are cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the production, and preferably secretion, of the polypeptide, followed by recovery of the desired product.
- 10 Furthermore, owing to the reduced activity of the metalloprotease, the recombinant polypeptide expressed by the host cell may be obtained as a precursor protein, i.e. a zymogen, a hybrid protein, a protein obtained as a pro sequence or pre-pro sequence, or in unmaturationed form.
- 15 The broth or medium used for culturing may be any conventional medium suitable for growing the host cell in question, and may be composed according to the principles of the prior art. The medium preferably contains carbon and nitrogen sources and other inorganic salts. Suitable media, e.g. minimal or complex media, are available from commercial suppliers, or may be prepared according to published protocols.
- 20 With regard to yeast host cells, it is often advantageous to produce heterologous polypeptides in a diploid yeast culture, because possible genetical defects may become phenotypically expressed in a haploid yeast culture, e.g. during continuous fermentation in production scale, and because the yield may be higher. The production
- 25 of recombinant polypeptides in yeast host cell is described in *Molecular Genetics of Yeast: A Practical Approach*, Ed. J.R. Johnston, IRL Press (1994) which is incorporated herein by reference.
- After cultivation, the protein is recovered by conventional methods for isolation and
- 30 purification proteins from a culture broth. Well-known purification procedures include separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, and chromatographic methods such as e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, etc.
- 35 The present invention is exemplified by demonstrating that the total amount of CCK and proBNP is increased about 60% about 100%, respectively when compared to the



non-modified host cell. The examples thus demonstrate that the host cells of the present invention are able to increase the production of diverse proteins.

Further, the examples disclose that additional disruption of other proteases enhance  
5 the production of the protein of interest, for example disruption of both *KEX2* and *CYM1* results in an additive effect in the yield in the production of CCK (nearly 100%).

Further, it will be understood by the skilled addressee that special amino acids within  
10 some of the motifs described, particularly His, Glu, Asp or Lys, are essential to the function of the metalloprotease by functioning as metal ligands.

Furthermore, it will be recognised that the metalloproteases of the present invention  
15 have been annotated widely in the literature as family members of the pitrilysin family, insulysin family, insulinase family, inverzincin family and M16 subfamily of clan ME.

Thus, it will be understood that any feature and/or aspect discussed above in  
connection with any of these different family annotations apply by analogy to the  
metalloprotease described herein, which all include the HXXEH motif.

20 However, please note that pitrilysin (without family) in itself refers to a specific member of clan ME of metalloproteases in *E. coli*.

## Examples

### Materials and Methods

#### Yeast strains and growth conditions

25 The yeast strains used are listed in Table I. Construction of strains were carried out using either the two step gene disruption technique (Rothstein, 1991) or the PCR based method by (Brachmann et al., 1998). Media were purchased from Difco, amino acids and other supplements from Sigma-Aldrich. Yeast cells were grown at 30°C in YPD (1%  
30 yeast extract, 2% peptone and 2% dextrose) or synthetic complete media (SC) based on yeast nitrogen base with ammonium sulfate, succinic acid, NaOH and appropriate amino acids. Transformations with either linear DNA or plasmids were performed using the modified lithium acetate procedure as described (Gietz et al., 1995). Analysis of heterologous expressed CCK was performed from yeast growing in exponential phase  
35 due to the consistency in CCK-22 biosynthesis, in contrast to the results from yeast within the stationary phase (Fig. 2). ProCCK processing was analysed from cell extract

and media of 5 A<sub>600</sub> units of cells per ml synthetic complete media. Cell growth was followed by the absorbance at 600 nm.

**Table 1.** *S. cerevisiae* strains used in this study. Null mutants of putative  
5 metalloproteases are named by the ORF in the genotype and (\*) represents mitochondrial proteases.

Strain	Genotype	Source
BY4705	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG his3<math>\Delta</math>200 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math></i>	(Brachmann et al., 1998)
LJY13	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG his3<math>\Delta</math>200 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> yps1::TRP1</i>	This study
LJY14	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG his3<math>\Delta</math>200 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> yps1::TRP1 yps3::LEU2</i>	This study
LJY15	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG his3<math>\Delta</math>200 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> yps1::TRP1 yps3::LEU2 yps2::URA3</i>	This study
BJ2168	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2</i>	(Jones, 1991)
LJY21	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 kex1::LEU2</i>	This study
LJY22	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 kex1::LEU2 kex2::TRP1</i>	This study
LJY23	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 kex2::TRP1</i>	This study
LJY122	<i>MAT<math>\alpha</math> ape1::KANMX ape2::LYS2 his3<math>\Delta</math> leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math></i>	This study
LJY123	<i>MAT<math>\alpha</math> ape1::KANMX ape2::LYS2 ape3::LEU2 his3<math>\Delta</math> leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math></i>	This study
LJY201	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 axl1::LEU2</i>	This study
LJY202	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 ste24::LEU2</i>	This study
LJY203	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 prd1:LEU2</i>	This study
LJY204	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 yil108w::LEU2</i>	This study
Y15298	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> ste23::KANMX</i>	Euroscarf
Y11874	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> aap1::KANMX</i>	Euroscarf
Y10148 (*)	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> afg3::KANMX</i>	Euroscarf
Y14953	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> ape1::KANMX</i>	Euroscarf
Y16224 (*)	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> rca1::KANMX</i>	Euroscarf
Y14984 (*)	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> mip1::KANMX</i>	Euroscarf
Y17144	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> yme1::KANMX</i>	Euroscarf
Y13211	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> ybr074w::KANMX</i>	Euroscarf
Y13801	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math> lys2<math>\Delta</math> ura3<math>\Delta</math> ydl104c::KANMX</i>	Euroscarf

Y11941	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yhr113w::KANMX</i>	Euroscarf
Y11960	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yhr132c::KANMX</i>	Euroscarf
Y12296	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yil137c::KANMX</i>	Euroscarf
Y15370	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ynl045w::KANMX</i>	Euroscarf
10864B	<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>851 leu2-<math>\Delta</math>1 his3<math>\Delta</math>200 lys2<math>\Delta</math>202 ykr035c-ykr038c::URA3</i>	Euroscarf
Y11749	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yol057w::KANMX</i>	Euroscarf
Y16248	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yol098c::KANMX</i>	Euroscarf
10231B	<i>MAT<math>\alpha</math> his3<math>\Delta</math>200 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 ura3-52 yol154w(4,744)::KANMX</i>	Euroscarf
Y14266	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ydr430c::KANMX</i>	Euroscarf
LJY430	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 ydr430c::LEU2</i>	This study
LJY432	<i>MAT<math>\alpha</math> prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2 kex2::TRP1 ydr430c::LEU2</i>	This study

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## DNA extraction and amplification

Yeast genomic DNA was isolated as described (Philippsen et al., 1991). Polymerase chain reaction (PCR) was performed using either *Pwo* polymerase or the enzyme cocktail based on *Taq*, *Pwo* and *Pfu* polymerase (Expand long range PCR kit, XL-PCR) both from Roche. All PCR products were visualised by agarose gel-electrophoresis and PCR products either purified from the gel using the gel-extraction kit (Qiagen) or from the reaction mixture by PCR purification spin columns (GENOMED). PCR based one step gene disruption was performed using 50 ng of plasmid from the pRS400 series (Brachmann et al., 1998) as template. Amplification of the marker was performed with oligonucleotides having 20 nucleotides towards the plasmid and additional 50 nucleotides flanking the target gene (Table 2). All other DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989).

## Plasmid constructions

Expression of proCCK was performed in pRS426 [2 $\mu$  *URA3*] (Brachmann et al., 1998) using the phosphoglycerate kinase promoter (*PGK1p*) and terminator (*PGK1t*). The *PGK1* promoter was amplified with *PGK1p* 5'*HindIII* and *PGK1p* 3'*MCS* (Table 2) using 100 ng of genomic yeast DNA as template and subsequently cloned into pGEM-11 (Promega) in the *HindIII* and *SacI* restriction enzyme sites. The terminator was amplified with *PGK1t* 5'*BglII* and *PGK1t* 3'*SacI* (Table 2) and ligated into the plasmid containing the promoter at the *SacI* and *EcoRI* restriction enzyme sites. This construct, pGEM-11 *PGK1pMCS**PGK1t* then contained the *PGK1*-promoter, a multiple cloning site (*MCS*) with the restriction enzyme sites *EcoRI*, *BamHI*, *XbaI* and *BglII* followed by the *PGK1* terminator. The preproM $\alpha$ 1p-proCCK fusion (Rourke et al., 1997) (Fig. 1) was subcloned into the *EcoRI* and *XbaI* sites of pGEM-11 *PGK1pMCS**PGK1t* and finally the entire gene was cloned into pRS423 as well as pRS426 to complete the yeast CCK expression constructs, pRS423 preproM $\alpha$ 1p-proCCK and pRS426 preproM $\alpha$ 1p-proCCK respectively. Expression of *CYM1* on a multi copy plasmid was constructed by amplification of the open reading frame (ORF) of *CYM1* and additional 926 bp at the 5' end and 703 bp at the 3' end. The amplification was carried out by XL-PCR using 100 ng of genomic yeast DNA and the oligonucleotides, *CYM1* 5'*ApaI* and

**Table 2. Oligonucleotides used.**

Oligo	Oligonucleotide sequence (5'-3')	Purpose
<i>PGK1p5'HindIII</i>	AATAGAAGCTTGTGCGACTGATCTATCCAAAAGT (SEQ ID NO: 16)	Expression construct
<i>PGK1p3'MCS</i>	AAAAGAGCTCGGCCAGATCTTCTAGAGGATCCAA GAATTCTGTTTTATATTTGTTGTAAAAAGTAG (SEQ ID NO: 17)	Expression construct
<i>PGK1t5'BglII</i>	TTTTGAATTCCAAGATCTCCCATGTCTCTACTGGTGG (SEQ ID NO: 18)	Expression construct
<i>PGK1t3'SacI</i>	CCCCGAGCTCGTCGACCCTTCTCGAAAGCTTTAACGAAC GC (SEQ ID NO: 19)	Expression construct
5'MF $\alpha$ 1- <i>EcoRI</i>	TTTTGAATTCAAAGAATGAGATTTCTTCAATTTTACTG CAG (SEQ ID NO: 20)	preproMf $\alpha$ 1p-proC CK
CCK3'- <i>XbaI</i>	TTTTTCTAGACTAGGAGGGTACTCATACTCCTCGGC (SEQ ID NO: 21)	preproMf $\alpha$ 1p-proC CK
CCK-22 K→A (S)	CGAATGTCCATCGTTGCGAACCTGCAGAACCTG (SEQ ID NO: 22)	Lys <sup>61</sup> →Ala <sup>61</sup> mutation
CCK-22 K→A (AS)	CAGGTTCTGCAGGTTCTTAACGATGGACATTCG (SEQ ID NO: 23)	Lys <sup>61</sup> →Ala <sup>61</sup> mutation
CCK-22 K→R (S)	CGAATGTCCATCGTTAGGAACCTGCAGAACCTG (SEQ ID NO: 24)	Lys <sup>61</sup> →Arg <sup>61</sup> mutation
CCK-22 K→R (AS)	CAGGTTCTGCAGGTTCTTAACGATGGACATTCG (SEQ ID NO: 25)	Lys <sup>61</sup> →Arg <sup>61</sup> mutation
CCK-22 seq	TCGCAGAGAACGGATGGC (SEQ ID NO: 26)	Sequencing
<i>CYM15'ApaI</i>	TTTTGGGCCCTTCATGGTGATACGGTATCTCTTGGC (SEQ ID NO: 27)	Cloning of <i>CYM1</i>
<i>CYM13'XhoI</i>	TTTTCTCGAGAAGGTGGAACATACTGCCCTGGGATGG (SEQ ID NO: 28)	Cloning of <i>CYM1</i>
<i>KEX25'</i>	TTTTGAGCTCGTTTAGGAAACGTCCTTGCGGAGATGC (SEQ ID NO: 29)	Cloning of <i>KEX2</i>
<i>KEX23'</i>	TTTTTCTAGACACTGCGAATCCATGGTATAAACCAAAACC (SEQ ID NO: 30)	Cloning of <i>KEX2</i>
<i>KEX2DC5'</i>	GTCGTTGTTTCATGGACATACCTCC (SEQ ID NO: 31)	Control of $\Delta$ kex2
<i>KEX2DC3'</i>	TACAAATGTTCTTCTGCCATTTCTGG (SEQ ID NO: 32)	Control of $\Delta$ kex2
<i>TRP15'NdeI</i>	GGTTCATATGCGCCGGAGCTCCTCGACAGCAG (SEQ ID NO: 33)	Cloning of <i>TRP1</i>
<i>TRP13'AvrII</i>	GGTTCCTAGGATCCGCAAGTTTGATTCCATTGCGGTG	Cloning of <i>TRP1</i>

	(SEQ ID NO: 34)	
<i>KEX15'</i> GD400	TTAAAGAGTACCTTGGCTATAGAATACCGTAGAGATAAA GACCTGAATAGAGATTGTACTGAGAGTGCAC	<i>KEX1</i> deletion
	(SEQ ID NO: 35)	
<i>KEX13'</i> GD400	AGGTATTATAACTATTTTTCTGTATTTTTTATATATTTTTAT TTGCCAAGCTGTGCGGTATTTACACCG	<i>KEX1</i> deletion
	(SEQ ID NO: 36)	
<i>KEX15'</i> DC400	CTTTGGTTAAAGAGTACCTTGGC (SEQ ID NO: 37)	Control of $\Delta kex1$
<i>KEX13'</i> DC400	TACTACGAAAAGCGTGTGCGAGG (SEQ ID NO: 38)	Control of $\Delta kex1$
	TAGAAGGCTACTCAAAAGAATAAAGTTACTATAAAATATA CTGCGGTATATAGATTGTACTGAGAGTGCAC	<i>CYM1</i> deletion
	(SEQ ID NO: 39)	
<i>CYM13'</i> GD400	GATCGGCAAGAACTTTGAAGCAGTATATTTACAGGATT AAATTATATATCTGTGCGGTATTTACACCG	<i>CYM1</i> deletion
	(SEQ ID NO: 40)	
<i>CYM15'</i> DC400	CGGAGGGGCTCTATGATAAAGG (SEQ ID NO: 41)	Control of $\Delta cym1$
<i>CYM13'</i> DC400	GAGTAACTAGGGCTTCTCTTCCC (SEQ ID NO: 42)	Control of $\Delta cym1$

*CYM1* 3'*XhoI* (Table 2). The PCR product was purified on spin columns and subsequently cloned into the *ApaI* and *XhoI* restriction enzyme sites of pRS425.

5

The Lys<sup>61</sup> residue, believed to be crucial for the proteolysis of proCCK to release CCK-22, was exchanged by Ala by site-directed mutagenesis (Horton et al., 1993). The exchange was performed by PCR using the *Pwo* polymerase (Boehringer Mannheim), where two products were amplified with the oligonucleotides sets, *PGK1p5'* *HindIII* / CCK-22 K→A (antisense) and CCK-22 K→A (sense) / *PGK1t3'* *SacI* (Table 2) and 50 ng of pRS426 preproMf $\alpha$ 1p-proCCK as template to each reaction. The two products were subjected to agarose gel-electrophoresis and approximately 1 mm<sup>2</sup> of each product where cut out and used directly as template in a third PCR reaction. In this reaction the full-length cDNA encoding the fusion protein was amplified using *PGK1p5'* *HindIII* and *PGK1t3'* *SacI* (Table 2). The PCR product was subcloned into pCR-Blunt II (Invitrogen) and sequenced with the CCK specific primer, CCK-22 seq. Finally the *PGK1p* preproMf $\alpha$ 1p-proCCK (K→A) *PGK1t* product was cloned into the *HindIII* and *SacI* sites of pRS426 to construct the expression plasmid, proCCK (K→A). Substitution of Lys with Arg was performed as described above by exchanging the CCK specific primers with

CCK-22 K→R (antisense) and CCK-22 K→R (sense) to construct the proCCK (K→R) vector.

#### Strain construction

5

Construction of a partial *KEX2* disruption was performed in BJ2168 by amplification of the entire *KEX2* gene with 1000 bp on each site of the ORF by XL-PCR using 100 ng of genomic yeast DNA and the oligonucleotides, *KEX2* 5'*Sac*I and *KEX2* 3'*Xba*I (Table 2). The PCR product was purified on spin columns and cloned into pCR-Blunt II (Invitrogen). Amplification of *TRP1* was performed by XL-PCR introducing an *Nde*I site 925 bp 5' to the ORF and an *Avr*II site 212 bp 3' to the stop codon using *TRP1* 5'*Nde*I and *TRP1* 3'*Avr*II (Table 2). The PCR product was purified and subcloned into the *Nde*I and *Avr*II sites of *KEX2* eliminating 2018 bp of *KEX2* and 170 bp of the promoter. The *kex2::TRP1* construct was excised from pCR-Blunt II using the *Not*I and *Spe*I restriction enzymes and subsequently transformed into BJ2168. Transformants were selected on SC-Trp plates followed by a colony PCR screen to test for correct integration using oligonucleotides that cover the entire marker plus an additional 1200 bp on each site of *KEX2*, *kex2* DC5' and *kex2* DC3' (Table 2). Construction of a *kex2* *kex1* strain was performed by the two step gene disruption technique (Rothstein, 1991) using the *LEU2* marker. Amplification of *LEU2* was performed by XL-PCR using 50 ng of pRS405 as template and *kex15'*GD400 and *kex13'*GD400 (Table 2). The PCR product was purified using PCR purification spin kit (GENOMED) and subsequently transformed into LJY23. Transformants were selected on SC-Leu plates and correct integration was tested by PCR-based colony screen using *kex15'*DC and *kex13'*DC (Table 2).

25

A *Δcym1::LEU2* (LJY430) strain in a BJ2168 background was constructed by the one step gene disruption technique as described above for the *Δkex1* strain using the oligonucleotides, *CYM15'*GD400 and *CYM13'*GD400 (Table 2) for gene disruption and *cym15'*DC and *cym13'*DC (Table 2) for disruption control. All null mutants created by this method were prepared with oligonucleotides designed towards the 50 bases adjacent to the 5' and 3' UTR with a specific 3' end to the pRS400 series of vectors containing various markers (Brachmann et al., 1998). Transformants were selected on appropriate agar plates followed by a colony PCR screen to test for correct integration using oligonucleotides that cover the entire marker plus an additional 200 bp on each site. Only the oligonucleotides that are not positioned as described above are shown in Table 2.

35



Gene deletions of *STE24*, *AXL1*, *PRD1* and *YIL108w* were made in BJ2168 using the PCR disruption technique (Brachmann et al., 1998) and pRS405 [*LEU2*] as template.

- The LJY123, which contain gene deletions of *APE1*, -2 and -3, was derived from Y14953 using PCR disruption technique (Brachmann et al., 1998). *APE2* was initially replaced with the *LYS2* (pRS317 [*cen*; *LYS2*]) where the PCR product was purified from agarose gel prior to transformation and *APE3* was substituted with the *LEU2* marker (pRS405 [*LEU2*]).
- 10 The *yps1 yps2 yps3* triple mutant (LJY15) was constructed in BY4705 using the PCR disruption technique (Brachmann et al., 1998). The ORF of *YPS1* were initially deleted by insertion of the *TRP1* locus (pRS404) to generate LJY13. This strain was then used as host for the deletion of *YPS3* by insertion of the *LEU2* marker (pRS405) and finally the *YPS2* was deleted by insertion of the *URA3* marker by amplification of pRS406
- 15 [*URA3*] to construct LJY15 (Table I).

#### CCK and *CYM1* expression

- Human proCCK was expressed as a fusion protein between the prepro leader sequence of yeast  $\alpha$ -mating factor and proCCK (preproM $\alpha$ 1p-proCCK). The fusion construct was
- 20 expressed on multi-copy plasmids, with constitutively gene transcription from the phosphoglycerate kinase promoter. "ProCCK expression" refers to expression using pRS426 preproM $\alpha$ 1p-proCCK, which was used in all yeast strains with exception of BY4705 and the isogenic yapsins deletion strains, where proCCK was expressed from pRS423 preproM $\alpha$ 1p-proCCK. *CYM1* expression was driven by its own promoter.
- 25 Plasmid constructs, and oligonucleotides used are listed in Table 2.

#### Enzymatic treatment

- Trypsin treatment was performed using 1 mg/ml Trypsin (Worthington Biochemical Corporation) in a 50 mM sodium phosphate buffer (pH 7.5) for 30 min at RT and
- 30 terminated by immersion into boiling water for 10 min. Carboxypeptidase B (Boehringer Mannheim) treatment with a final concentration of 4  $\mu$ g/ml was performed in 0.1 mM sodium phosphate buffer (pH 7.5) at room temperature for 30 min. The reaction was terminated by immersion into boiling water for 10 min.

### Gel chromatography

Yeast transformants grown to late exponential phase were centrifuged at 15000 *g* to collect the cells and 500  $\mu$ l of the medium was loaded directly onto a Sephadex G-50 superfine (Pharmacia) column (1 $\times$ 100 cm) at 4°C. The sample was eluted in VBA buffer (20 mM barbital buffer, 0.11% bovine serum albumin and 0.6 mM thiomersal) at a flow rate of 3.5 ml/h and fractions were collected every 17 min. Calibrations were performed by including  $^{125}$ I-albumin ( $V_0$ ) and  $^{22}$ NaCl ( $V_t$ ). The elution constants  $K_d$  of peaks eluting at  $V_e$  are calculated as  $K_d = (V_e - V_0)/(V_t - V_0)$ .

### Radio-immunoassay

Two different antisera were used to determine the amount of processed cholecystokinin. Ab 89009 (Paloheimo et al., 1994) is specific for the N-terminus of CCK-22 and Ab 7270 (Hilsted et al., 1986) is specific for Gly-extended CCK. The fraction of CCK processed to CCK-22 is calculated by division of the Immuno-reactivity measured with Ab 89009 with the amount measured with the same antibody after the sample was treated with trypsin to measure the total amount of N-terminal extended CCK-22.

### Yeast extract and protease assay

Ten  $A_{600}$  units of yeast cells growing in exponential phase were sedimented by centrifugation at 3000 *g* for 5 min, washed once in 25 ml H<sub>2</sub>O and transferred to a 2 ml Eppendorf tube. An equal amount of acid washed glass beads (Sigma-Aldrich) was added followed by 200  $\mu$ l of 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 4.5) including various inhibitors (150  $\mu$ M Bestatin, 30  $\mu$ M E-64, 10  $\mu$ M Leupeptin, 1  $\mu$ M Pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM 1,10-orthophenanthroline or 1 tablet complete inhibitor with or without EDTA per 2.5 ml 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (Boehringer Mannheim)). The cells were broken by vortexing 3 $\times$ 20 sec and the extracts were clarified by centrifugation at 15000 *g* for 10 min. All steps were carried out at 4°C. The protease assay was performed using 20 pmol synthetic amidated CCK-33 (Peninsula Laboratorie Europe, Merseyside, England) or Ac-CCK-33-Gly (Cambridge Research Biochemicals, Stockton, England) as substrate, 20  $\mu$ l yeast extract, various inhibitors and activators in a total volume of 30  $\mu$ l. The mixture was incubated at 30°C

for 1 h and the reaction terminated by adding 500  $\mu$ l VBA buffer followed by immediate immersion into a boiling water bath for 10 min.

#### Protease assay using metalloprotease deficient strains

5

The assay was performed as described above, but with addition of 1 mM Bestatin and 1 mM  $Mn^{2+}$  to decrease N-terminal degradation.

#### Protease assay using intact yeast cells

10

Five  $A_{600}$  units of exponential growing cells were sedimented, washed once in 5 ml  $H_2O$  and once in SC media (pH 6.0), before the cells were resuspended in 25  $\mu$ l SC media. The protease assay was performed by addition of 20 pmol synthetic Ac-CCK-33-Gly as substrate and the mixture incubated with gentle shaking at 30°C for 1 h. The reaction was terminated by addition of 500  $\mu$ l VBA and the cells removed by centrifugation before the supernatant was immersed into boiling water for 10 min.

#### Analysis of secreted CCK by MALDI-TOF

20 Fifty  $A_{600}$  units of CCK transformed yeast cells were subjected to 25 ml of fresh media, followed by inoculation for 3 h. Cells were removed by centrifugation at 15000  $g$  for 10 min and 500  $\mu$ l of media was concentrated and desalted by reverse phase using a ZipTip  $C_{18}$  column (Millipore). The peptides were eluted with 10  $\mu$ l 50% acetonitrile. The purified peptides were analysed in a Matrix Assisted Laser Desorption/Ionization time-of-flight mass spectrometer (Biflex, Bruker-Franzen, Bremen, Germany) operated in the reflected mode using time lag focusing (delayed extraction). For analysis, 0.5  $\mu$ l of the sample was mixed with 0.5  $\mu$ l matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid in acetonitrile/methanol, Hewlett Packard). Then 0.5  $\mu$ l of the mixture was applied to the probe and allowed to dry before introduction into the mass spectrometer.

30

#### Statistical analysis

Statistical calculations were performed using an unpaired students t-test to analyse whether the change in proCCK expression or the fraction of mature CCK-22 between wild type yeast expressing proCCK and mutants isogenic to the wild type strain can be considered to be statistically significant. The P-value calculated for CCK-22 processing between yapsin mutants are comparisons of BY4705 and each mutant, whereas the

35

brackets represent comparisons between BY4705 *yps1* and BY4705 *yps1 yps3* and, BY4705 *yps1 yps3* and BY4705 *yps1 yps2 yps3*, respectively.

Expression of proBNP in *Saccharomyces cerevisiae* – Construction of the *yps1* mutant

#### 5 Cloning of preproBNP

Messenger RNA was isolated from a 500 mg Biopsy from human heart using the Quickprep Micro mRNA purification Kit (Amersham Pharmacia Biotech). First strand cDNA was prepared from 2 µg mRNA in a reaction containing, 2.5 µl 10× first strand  
10 buffer (Promega), 2.5 µl 100 mM DTT, 2.5 µl 10 mM dNTP, 2.5 µl Na pyrophosphate, 10 pmol Oligo(dT)<sub>18</sub>, 10 units reverse transcriptase, AMV (Promega), and H<sub>2</sub>O to 25 µl. Messenger RNA and Oligo(dT)<sub>18</sub> was heated to 70°C for 5 min cooled on ice for 5 min prior to cDNA synthesis. The first strand cDNA synthesis was performed at 42°C for 60 min.

15

The cDNA encoding preproBNP was amplified using Pwo polymerase (Roche), 1 µl 1. strand cDNA, 5 µl 10× Pwo buffer included MgCl<sub>2</sub> (Roche), 5 µl 2.5 mM dNTP, 30 pmol of each primer (BNP5'*Eco*RI and BNP3'*Xba*I). The PCR product encoding preproBNP was cloned in pBluescript II (Stratagene). All subsequent PCR reactions were performed as  
20 described above.

The fusion between the cDNA's encoding the preprosequence of the α-mating factor and proBNP was performed using overhang extension PCR, where two PCR reactions were set up. One using 50 ng of pRS426 preproMfα1p-proCCK as template,  
25 MFα15'*Eco*RI and MF1BNP (AS) as primers and a second with 50 ng of preproBNP cloned in pBluescript and the primers, MF1BNP (S) and BNP3'*Xba*I. In the third PCR reaction, approximately 50 ng of each PCR products were purified from agarosegel from the the two initial PCR using the gel-extraction kit (Qiagen) and used as template with the two oligonucleotides, MFα15'*Eco*RI and BNP3'*Xba*I. The preproMfα1p-proBNP  
30 encoding construct was subcloned in pCR-Blunt II (Invitrogen) and sequenced with vector specific oligonucleotides prior to subcloning into the *Eco*RI and *Xba*I sites of pGEM-11 *PGK1pMCSPGK1t*. Finally the entire gene was cloned into pRS426 to complete the yeast proBNP expression constructs, pRS426 preproMfα1p-proBNP.

35 Furthermore, two additional constructs have been made, in which the proBNP fragment (1-76) has been removed. These constructs are similar to do the preproMfα1p-proBNP, but do only synthesise BNP-32. In the first construct, the Kex2p cleavage site and the

- spacer peptide of the preproMf $\alpha$ 1p has been sustained (KREAEA)(Figure 14B), whereas in the other construct, the spacer peptide has been removed (Figure 14C). Analysis of the BNP-32 expression from wild type yeast and the isogenic *CYM1* disruptant will be analysed by RIA's using the Shionoria-BNP system from Electra-Box Diagnostica ApS.
- 5 This assay is specific for BNP-32.

#### *Expression of proCCK, proBNP and Cym1p*

- Human proCCK was expressed as a fusion protein between the prepro leader sequence of yeast  $\alpha$ -mating factor and proCCK (preproMf $\alpha$ 1p-proCCK). The fusion construct was expressed on multi-copy plasmids, with constitutively gene transcription from the phosphoglycerate kinase promoter. "ProCCK expression" refers to expression using pRS426 preproMf $\alpha$ 1p-proCCK, which was used in all yeast strains with exception of BY4705 and the isogenic yapsins deletion strains, where proCCK was expressed from pRS423 preproMf $\alpha$ 1p-proCCK. Human proBNP was also expressed as a fusion protein between the prepro leader sequence of yeast  $\alpha$ -mating factor and proBNP (preproMf $\alpha$ 1p-proBNP) (Figure 14A). *CYM1* expression was driven by its own promoter. Plasmid constructs, and oligonucleotides used are listed in Table 2.
- 10
- 15

#### 20 *BNP radioimmunoassay*

Antibody 98192 is specific for the N-terminus of proBNP (Gøtze et al., 2002).

#### *Chromatography*

- 25 FPLC chromatography was performed on a Superdex 200 column on a Äkta purifier (Amersham Pharmacia Biotech). In the 50 mM Na-phosphate buffer, 100 mM NaCl and 6 M Guanidin were included.

#### *Strain construction*

- 30 A  $\Delta yps1::TRP1$  (LJY440) and a  $\Delta cym1::LEU2 \Delta yps1::TRP1$  (LJY431) strain in a BJ2168 background were constructed by the one step gene disruption technique as described above using the oligonucleotides, *YPS15'*GD400 and *YPS13'*GD400 (Table 2) for gene disruption. The PCR product was transformed into BJ2168 and LJY430. Verification of the correct integration of the disruption cassette was analysed by PCR using *yps15'*DC and *yps13'*DC (Table 2).
- 35

Identification of the gene encoding the Cym1 orthologue in *Pichia pastoris* or *Pichia methanolica*

Identification of the unknown genes from *Pichia pastoris* and *Pichia methanolica*

- 5 encoding the Cym1p orthologues of *Saccharomyces cerevisiae* will be carried out in similar manner, using the same set of degenerated primers mentioned below. *Pichia pastoris* and *Pichia methanolica* will be referred to as *Pichia* in the following text.

- By alignment of the orthologous Cym1 proteins of *Saccharomyces kluyveri* and  
10 *Schizosaccharomyces pombe* to Cym1p from *Saccharomyces cerevisiae*, there was identified a number of identical amino acid sequences. From these sequences it is possible to synthesize degenerated oligonucleotides (Table 3) that will bind to the complementary DNA strands of *CYM1* in all three species, and thus to the *CYM1* gene of *Pichia*. Amplification of the genomic sequence will initially be carried out by using high  
15 quality genomic DNA as template, *Pichia-CYM1-Ia* and *Pichia-CYM1-Ib* and the *Pwo* polymerase (Roche). The amplified sequence with an expected size of approximately 525 bp will be cloned in pBlunt or a similar vector and sequenced with vector specific primers. If no band appear from the initial amplification, a second round of PCR will be performed with the two nested primers, *Pichia-CYM1-IIa* and *Pichia-CYM1-IIb* using 1 µl  
20 of the first PCR reaction as template. The expected product is approximately 270 bp and will be cloned in pBlunt and sequenced with M13 forward and M13 reverse primers. From the obtained sequence there will be synthesized sequence specific primers, two nested sense and two nested antisense specific primers. Using one of the sense primers it is possible to obtain a PCR product with *Pichia-CYM1-IIIb* using high  
25 quality genomic DNA as template. This product of ~2100 bp will be cloned and sequenced. If it fails to produce a band of ~2100 bp, it would be necessary to isolate mRNA from *Pichia*, produce double stranded cDNA and ligate adaptors to the ends as described by the manual to the Clontech Marathon cDNA Amplification Kit (BD (Becton, Dickinson and Company)). Using the two sequence specific sense primers it is possible  
30 to obtain the 3' end of the mRNA of approximately 2600 bp and the sequence specific antisense primers to amplify the 5' end including the sequence encoding the hypothetical active site, HXXEH motif. Synthesis of sequence specific oligonucleotides from the 5' and 3' untranslated region, full-length cDNA encoding the Cym1 orthologue in *Pichia* can be cloned.

Table 3

	Amino acid sequence	K Y P V R D P
	Oligo <i>Pichia</i> -CYM1-Ia	5' AARTAYCCXGTXMGXGAYCC 3'
5		
	Amino acid sequence	H P S N A K
	Oligo <i>Pichia</i> -CYM1-Ib	3' GTRGGXWSXTTTRCGXTTY 5'
	Amino acid sequence	D P F F K M
10	Oligo <i>Pichia</i> -CYM1-IIa	5' GAYCCXTTYTTTYAARATG 3'
	Amino acid sequence	G V V Y N E M
	Oligo <i>Pichia</i> -CYM1-IIb	3' CCXCAXCAXATRTTRCTYTAC 5'
15	Amino acid sequence	E K G G A Y G
	Oligo <i>Pichia</i> -CYM1-IIIb	3' CTYTTYCCXCCXCGXATRCC 5'

X-Inosine, degenerated oligonucleotides follow the International Union of Biochemistry (<http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html>).

20

#### Genedisruption of Cym1 orthologue in *Pichia pastoris* or *Pichia methanolica*

The sequence encoding the Cym1 orthologue in *Pichia* should be cloned in a vector like pBluescript-II in *Hind*III and *Sac*I or a similar vector, if these are not present in the

25 *ORF*. Insertion of the *ORF* in *Hind*III and *Sac*I sites removes most of the multiple cloning sites from the vector, which ease the possibility to find restriction enzyme sites that are only present in the *ORF*. Cloning of the *KanMX* cassette within the *ORF*, preferentially so that 1000 bp of the *Pichia CYM1* are present on each site of the *KanMX* cassette creates the *Pichia CYM1* disruption cassette. This construct can then be

30 amplified by PCR, using primers at specific for the 5' and 3' end of the *Pichia CYM1* gene. Transformation of the PCR product into strains of *Pichia* followed by selection of transformants on YPD plates containing 100 µg/ml geneticin (G-418). Verification of the correct integration into the *Pichia* genome should be tested by colony PCR, using *Pichia* sequence specific *CYM1* primers that binds 5' and 3' to the *KanMX* cassette. From the

35 size of the PCR product it is possible to distinguish whether the integration event is correct.

#### Expression of foreign proteins and peptides in *Pichia pastoris*

For expression of peptides one could use the expression vector, pPIC $\alpha$  (inducible expression) or pGAPZ $\alpha$  (constitutive expression) both from Invitrogen. Both these  
5 vectors use the preprosequence of the  $\alpha$ -mating factor from *Saccharomyces cerevisiae* to direct the fusion peptide through the secretory pathway. Within the Golgi apparatus the preprosequence of the  $\alpha$ -mating factor is removed and the peptide of interest released to the media. If it's proteins that should be expressed, both vectors metioned  
10 pPICZ and pGAPZ), where the heterologus expressed protein is cytosolic located and can be isolated from intact cells.

#### Expression of forelgn proteins and peptides in *Pichia methanolica*

15 Expression of proteins and peptides in *Pichia methanolica* is performed in a similar manner as in *Pichia pastoris*, where plasmids are avaiable both for *intracellular* expression and for secretion to the media. *Intracellular* expressed proteins can be cloned into pMET (Invitrogen) and for secretion in pMET $\alpha$  (Invitrogen). The pMET $\alpha$  contain the preprosequence of the  $\alpha$ -mating factor from *Saccharomyces cerevisiae* as  
20 used for expression in *Pichia pastoris*. Expression is induced by methanol in this system.

### Results

#### The influence of growth conditions on the CCK-22 processing

25 The intra- and extracellular fraction of CCK-22 was measured from BJ2168 expressing proCCK. The intracellular fraction remained unaltered whether the cells were in exponential growth or had reached stationary phase (Fig. 2). However, the relative amount of secreted CCK-22 changed dramatically when the cells reached stationary  
30 phase. During exponential growth the fraction of CCK-22 was 23%, but in the stationary phase (after 270 min) the fraction increased to 37% (Fig. 2). Hence, for the experiments described herein only exponentially growing cells were used.



The significance of the Lys residue in the release of CCK-22

To evaluate the role of the Lys residue in proteolysis, transformants of BJ2168 with the two expression constructs, proCCK and proCCK (K→A) were grown to late exponential phase and the culture media collected. The media from each strain was subjected to gel chromatography and the content of Gly-extended CCK in the collected fractions where measured with Ab 7270. CCK from the wild type media eluted in two major peaks at  $K_d = 0.8$  and 1.1 (Fig. 3 A) in accordance with the previously established elution positions for CCK-22-Gly and CCK-8-Gly (Cantor et al., 1987; Rourke et al., 1997), while the proCCK (K→A) only gave rise to CCK-8-Gly and a larger form eluting at a  $K_d = 0.6$  (Fig. 3 C). The two peaks eluting at  $K_d = 0.7$  and 0.8 for the wt construct (Fig. 3 B) correspond to C-terminally extended CCK-22 and CCK-22-Gly, respectively (Rourke et al., 1997), whereas no CCK-22 immuno-reactivity was observed in these positions for the proCCK (K→A) construct (Fig. 3 D). However, a small peak of immuno-reactivity was seen at  $K_d = 0.55$  which may be due to the slight cross reactivity of Ab 89009 with a larger unprocessed fragment (Paloheimo et al., 1994). To investigate the effect of substituting Arg for Lys, proCCK (K→R) was transformed into BJ2168. Media from transformants were analysed before and after tryptic cleavage. The fraction of proCCK processed to CCK-22 was similar to that seen for wild type CCK (Fig. 11).

#### Analysis of secreted CCK peptides by mass spectrometry

Media collected from BJ2168 transformed with proCCK were analysed by mass spectrometry (Figure 12). The fragments obtained correspond to the processing leading to CCK-39, CCK-22 and CCK-8. Two peptides were identified N-terminal of Lys<sup>61</sup> (Tyr<sup>45</sup>-Val<sup>60</sup>, 1805.0 Da and Tyr<sup>45</sup>-Lys<sup>61</sup>, 1932.2 Da). It appeared likely that the former was a carboxypeptidase degradation product of the latter. To elucidate this question and in an attempt to identify the C-terminal extended CCKs, the present inventors produced a disruption strain in which both *KEX2*, encoding the serine protease responsible for the processing to CCK-8, and the carboxypeptidase encoded by *KEX1* were mutated. Following transformation of proCCK into this *kex2 kex1* strain (LJY22) and subsequent analysis of the secreted peptides the inventors found only the peak corresponding to Tyr<sup>45</sup>-Lys<sup>61</sup>. The same pattern, with only the peak corresponding to Tyr<sup>45</sup>-Lys<sup>61</sup> was seen using single gene disruption of *KEX1* and *KEX2* to express proCCK (data not shown). Thus the Tyr<sup>45</sup>-Val<sup>60</sup> must be a degradation product in accordance with CCK-22 arising from cleavage after Lys<sup>61</sup>. Additional fragments were discovered by CCK

expression in the *kex2 kex1* strain corresponding to processing leading to CCK-61 (not identified in mammals), CCK-58, C-terminal extended CCK-39 and C-terminal extended CCK-22 (Figure 12), whereas none of the peptides corresponding to CCK-8 could be identified, in accordance with our previous work showing that Kex2p is responsible for this processing (Rourke et al., 1997).

#### Kex2p is involved in the biosynthesis of CCK-22

Previous analysis of CCK peptides secreted from a *kex2* strain as well as the results obtained by mass spectrometry indicate that the cleavage at Lys<sup>61</sup> releasing CCK-22 can occur without the involvement of Kex2p. However, the *kex2* strain shows a decrease in CCK-22 concentration. ProCCK was expressed both in the vacuole protease deficient and the isogenic *kex2* strain (BJ2168 and LJY23) and the processed intra- and extracellular fractions of CCK-22 from exponentially growing cells were measured. Approximately 28% of the intracellular CCK content was processed after Lys<sup>61</sup> in BJ2168, whereas only 6% was processed within the *kex2* strain. Analysis of secreted CCK peptides showed that the media collected from BJ2168 expressing proCCK contained approximately 20% CCK-22, whereas from the *kex2* mutant, the amount was reduced to 5%. These results indicate that Kex2p is involved in the processing leading to CCK-22. However, there are other proteases that can perform the cleavage at Lys<sup>61</sup>.

#### In vitro assay of Lys<sup>61</sup> cleavage

To investigate the nature of the protease(s) in addition to Kex2p that are able to perform the endoproteolytic cleavage after the single Lys<sup>61</sup> residue of proCCK, an *in vitro* assay was established using crude preparations from of *S. cerevisiae* and synthetic CCK-33 as substrate.

Using extract from the vacuole protease deficient strain, BJ2168, there was an extensive N-terminal degradation, and the recovery of measurable CCK was less than 10% of the control without yeast extract. Because the assay depends on the intact N-terminus of CCK-22 for the antibody to bind, the inventors created a strain where some of the known *S. cerevisiae* aminopeptidases were deleted. The Y14953 strain (*ape1*) was used as parental strain in which the *APE2* and *APE3* genes were also deleted. Using this LJY123 strain to prepare the cell extract there was a 2-3 fold better recovery of immuno-reactivity compared to the recovery seen with BJ2168.

Processing to CCK-22 depends on metal ions

The nature of the protease performing the cleavage of synthetic human CCK-33 to CCK-22 was analysed by inclusion of a number of different inhibitors with the extract  
5 from LJY123. The results showed that only the addition of a metal chelating agent inhibited proteolysis of CCK-33 to CCK-22 (Fig. 4 A).

The metal dependency of the protease was tested *in vitro*, after the activity initially was inhibited by addition of 1 mM EDTA. Reconstitution of the activity leading to maturation  
10 of CCK-22 was tested by addition of different divalent cations in 0.2 mM surplus. Addition of  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  could reestablish the protease activity, whereas  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  or  $\text{Mg}^{2+}$  had no effect (Fig. 4 B) in accordance with the properties of known metalloproteases, which are only activated by  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$ . Reactivation using increasing  $\text{Zn}^{2+}$  concentrations showed a biphasic pattern, with  $\text{Zn}^{2+}$  acting inhibitory at  
15 concentrations above 5 mM (data not shown).

The time course of CCK-cleavage by  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$  reactivated metalloproteases was analysed using cell extract from LJY123, after initial inhibition with 1 mM EDTA. Reactivation was performed by addition of 1.2 mM  $\text{Zn}^{2+}$  or  $\text{Mn}^{2+}$  followed by incubation  
20 for 30, 60 and 120 min. In this assay and the following *in vitro* protease assays the inventors used the N-terminal acetylated CCK-33-Gly (Ac-CCK-33-Gly) as substrate, which resulted in much slower non-specific degradation. Measurement of the CCK-22 immuno-reactivity before and after tryptic cleavage using Ab 89009 showed no difference in the activation potency between  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$  at 30 and 60 min, however  
25 after 120 min 10% more CCK-22 immuno-reactivity was measured using  $\text{Mn}^{2+}$  as activator compared to  $\text{Zn}^{2+}$  (Fig. 5). This increase in immuno-reactivity is probably due to an inhibition of degradation following addition of  $\text{Mn}^{2+}$  here as well as to the yeast cell extracts used in Table 4.

30 Table 4. Metalloproteases in *Saccharomyces cerevisiae*. Search performed in Swiss-Prot Sequence Retrieval System (SRS) <http://www.expasy.ch/>. Protease assay performed in two independent assays (A and B) using extracts from the metalloprotease deficient strains. The amount of CCK-22 is measured with Ab 89009 and the total amount of CCK is measured after tryptic cleavage with Ab 89009. Putative metalloproteases are marked  
35 with \*.

Name	Swiss-Prot acc #	ORF	CCK-22 [nM]	Total CCK [nM]	Fraction CCK-22
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			A <sub>1</sub>	B <sub>1</sub>	A <sub>2</sub>	B <sub>2</sub>	A <sub>1</sub> /A <sub>2</sub>	B <sub>1</sub> /B <sub>2</sub>
AAP1	<u>P37898</u>	YHR047c	3.2	3.2	36	32	0.09	0.10
AFG3	<u>P39925</u>	YER017c	2.8	2.4	23	24	0.12	0.10
APE1	<u>P14904</u>	YKL103c	4.4	3.7	34	28	0.13	0.13
APE2	<u>P32454</u>	YKL157w						
APE3	<u>P37302</u>	YBR286w						
DPP3	<u>Q08225</u>	YOL057w	4.3	4.6	31	35	0.14	0.13
LTA4	<u>Q10740</u>	YNL045w	3.9	4.0	32	29	0.12	0.13
MIP1	<u>P35999</u>	YKL134c	3.4	3.3	34	33	0.10	0.10
PRD1	<u>P25375</u>	YCL057w	2.4	2.8	28	26	0.09	0.11
QRI7*	<u>P43122</u>	YDL104c	2.8	2.9	23	24	0.12	0.12
RCA1	<u>P40341</u>	YMR089c	4.2	3.5	31	27	0.14	0.13
STE23	<u>Q06010</u>	YLR389c	2.6	2.3	25	20	0.10	0.12
STE24	<u>P47154</u>	YJR117w	3.4	2.8	19	17	0.18	0.16
YBS4*	<u>P38244</u>	YBR074w	2.6	2.7	27	29	0.10	0.09
YHR3*	<u>P38821</u>	YHR113w	2.5	2.4	26	26	0.10	0.09
YHT2*	<u>P38836</u>	YHR132c	3.8	3.3	34	32	0.11	0.10
YIK8*	<u>P40483</u>	YIL108w	5.7	5.2	43	39	0.13	0.13
YIN7*	<u>P40462</u>	YIL137c	3.5	2.8	23	20	0.15	0.14
YK18*	<u>P36132</u>	YKR038c	2.9	2.1	23	20	0.13	0.11
YME1	<u>P32795</u>	YPR024w	2.3	2.7	25	25	0.09	0.11
MAS2	<u>P11914</u>	YHR024c	ND, Lethal genes					
MAS1	<u>P10507</u>	YLR163c						
AXL1	<u>P40851</u>	YPR122w	3.5	3.4	31	30	0.11	0.11
CYM1*	<u>P32898</u>	YDR430c	0.6	0.4	42	39	0.01	0.01
YOJ8*	<u>Q12496</u>	YOL098c	3.6	3.8	35	34	0.10	0.12

### Extracellular yapsin activity

To investigate whether any protease activity is secreted or attached extracellularly to the plasma membrane, the protease activity was assayed in media and with intact  
5 yeast cells. No degradation of CCK-33 occurred after 1 h of incubation at 30°C using media from exponential growing LJY123 cells in accordance with earlier observations (Rourke et al., 1997). During incubation with intact yeast cells, cleavage to expose the N-terminus of CCK-22 could be measured (Fig. 6) however, this protease activity could not be abolished by the inhibitors investigated (data not shown). By using intact cells  
10 containing gene disruptions of *YPS1*, *YPS2* and *YPS3* (Table I) the fraction of processed CCK-22 decreases by deletion of each of the three aspartyl proteases compared to wild type cells (Fig. 6). These data show that the three proteases all have extracellular protease activity, which can cleave at Lys<sup>61</sup> in proCCK. Preliminary results indicate that gene disruption of *YPS7* decreases extracellular Lys<sup>61</sup> processing in amounts  
15 comparable to the *YPS1* deletion (unpublished results).

### Expression of proCCK in metalloprotease deficient strains

Based on previously described metalloproteases in *S. cerevisiae* with endoproteolytic  
20 activity (Adames et al., 1995; Schmidt et al., 2000), gene deletion strains of *AXL1* (LJY201) and *STE24* (LJY202) were initially prepared in BJ2168. ProCCK expression in these strains showed that proteolysis after Lys<sup>61</sup> was unchanged compared to wild type, and it was decided to test the remaining metalloprotease deficient strains LJY123, LJY203, LJY204 and the metalloprotease deficient strains obtained through Euroscarf  
25 (Table I) for their ability to secrete CCK-22 (mitochondrial peptidases were not included). The CCK-22 immuno-reactivity did not change significantly among the CCK producing metalloprotease deficient strains (data not shown), and no protease responsible for the processing of heterologous expressed proCCK to CCK-22 was identified by this approach.

30

*CYM1* encodes a protease that can release the free N-terminus of CCK-22

Cell extracts were prepared from each of the viable metalloprotease deficient strains and tested in the *in vitro* protease assay to investigate whether any reduction in  
35 proteolysis was measurable. In this assay 1 mM Mn<sup>2+</sup> and 1 mM bestatin were included prior to the addition of Ac-CCK-33-Gly, since it was found that the recovery was 80-90% compared to 30% without addition of these aminopeptidase inhibitors (data not

shown). Deletion of *CYM1* almost abolished the protease activity, whereas none of the other metalloprotease deficient strains showed a significant change in the biosynthesis of CCK-22 (Table 4).

- 5 Expression of *CYM1* on a multicopy plasmid increases the fraction of matured CCK-22 *in vitro*

To determine whether the amount of synthesized CCK-22 correlates with the amount of Cym1p *in vitro*, Cym1p was expressed on a multicopy plasmid and the fraction of  
10 synthesized CCK-22 analysed over time. Cell extract from BJ2168 transformed with pRS425 *CYM1* and the control transformed with the empty pRS425 vector were used in the *in vitro* protease assay with 1 mM Mn<sup>2+</sup> in which the reactions were terminated after 15, 30, 45 and 60 min. The CCK-22 immuno-reactivity was measured with Ab 89009 and the remaining CCK-33 was measured with the same antibody after tryptic  
15 cleavage (Fig. 7). Expression of *CYM1* on a multicopy plasmid enhanced the rate of CCK-22 production several fold. However, the inventors also observed an increased degradation of CCK-33 and CCK-22 (Fig. 7 B). When the same experiment was performed at pH 6.0 and pH 7.5, there was a dramatically increased degradation and after 30 min incubation the CCK immuno-reactivity was undetectable at pH 6.0 (data  
20 not shown). These results show that the Lys-specific cleavage in CCK-22 maturation *in vitro* is dependent on the amount of Cym1p.

Expression of proCCK in *cym1* mutant strain enhances CCK secretion

- 25 To elucidate the role of *CYM1* in the biosynthesis of CCK-22 *in vivo*, gene deletions of *CYM1* were prepared in the vacuole protease deficient strain, BJ2168, and isogenic *kex2* strain. Deletion of *CYM1* resulted in an approximately 40% increase in the total amount of proCCK within the cells (Fig. 8 A) accompanied by a similar decrease in CCK-22 independent of *KEX2* disruption (Fig. 8 C). Also the secreted amount of total  
30 CCK in the *cym1* strains increased with more than 60% (Fig. 8 B), but unlike the fractional decrease in intracellular CCK-22 there was an increase in the extracellular fractions of CCK-22 compared to vacuole protease deficient strain and the isogenic *kex2* strain (Fig. 8 D).

Expression of CCK K→A mutant leads to intracellular CCK accumulation comparable to the accumulation of wild type CCK in a *cym1* strain

- 5 The observations that a gene disruption of *CYM1* causes an increase in intracellular concentrations of CCK (Fig. 8 A) raise the question whether the proteolytic activity of Cym1p leads to degradation of CCK-22 prior to translocation into the ER. Therefore, the inventors examined the intracellular CCK content in strains expressing CCK where the maturation of CCK-22 has been eliminated by using the Lys<sup>61</sup> → Ala<sup>61</sup> mutant.
- 10 Transformants of this CCK mutant in the vacuole protease deficient strain, BJ2168 and the isogenic *cym1* strain were analysed using Ab 7270 after trypsin and carboxypeptidase B treatment and there was an increase in the intracellular CCK immuno-reactivity for this construct compared to expression of wild type CCK (Fig. 9). Mutant CCK (K→A) and wild type CCK transformants resulted in an increase in the
- 15 Intracellular proCCK concentration when expressed in BJ2168 and the *CYM1* disruption strain, respectively. The increase in intracellular proCCK was not additive showing that proteolytic activity of Cym1p leads to degradation of CCK-22 prior to translocation into the ER.
- 20 Expression of proCCK in aspartyl protease deficient strains
- The Lys<sup>61</sup>-specific cleavage of proCCK was analysed in null mutants of *YPS1*, -2 and -3, where the intra- and extracellular amount of CCK-22 was measured from exponentially growing cells of wild type yeast, BY4705 and the isogenic aspartyl protease deficient
- 25 strains transformed LJY13, -14 and -15 with proCCK. Both intra- and extracellular CCK immuno-reactivity of BY4705 was lowered more than 10 fold compared to the vacuolar protease deficient strain, BJ2168 (data not shown). The intracellular fraction of CCK-22 decreased significantly from approximately 28% in wild type cells to 17% in the *yps1* strain, whereas no additional decrease could be measured by gene disruptions of *YPS2*
- 30 and *YPS3* (Fig. 10 A). The extracellular fraction of CCK-22 did however show that Yps1p, Yps2p and Yps3p all are involved in the biosynthesis of CCK-22 and that the triple mutant reduced the fraction of CCK-22 to 2/3 compared to wild type yeast (Fig. 10 B).
- 35 *CYM1* disruption leads to a two-fold increase in the total amount of secreted wild type CCK as well as the CCK K→R mutant

To elucidate whether Cym1p cleaves C-terminally to a single Arg residue, the CCK (K→R) mutant was expressed in the vacuolar protease deficient strain, BJ2168 and the isogenic *cym1* strain. The concentration of both intra- and extracellular CCK was compared to wild type CCK expressed in these strains. The total amount of the mutated CCK (K→R) was increased both intra- and extracellular comparable to wild type CCK (Fig. 11). Both wild type CCK and the Lys<sup>61</sup>→Arg<sup>61</sup> mutant showed more than a two-fold increase in the measurable amount of extracellular CCK when expressed in the *cym1* strain (Fig. 11 B).

#### 10 Usage of Cym1p activity in the synthesis of peptides

Another aspect of the invention is to use the activity from Cym1p, either expressed from its own promoter or from a strong constitutive promoter such as *PGK1*, *ADH1* or *TPI1*, or the induceable *GAL1* promoter to produce an increased amount cytosolic Cym1p activity. As previously mentioned, the synthesis of CCK-22 is significantly increased when *CYM1* is transcribed from its own promoter on a 2μ plasmid (Fig. 7). Transcription can either be performed from a plasmid containing the promoter, *CYM1* and a terminator, or by introducing the desired promoter into the genome by heterologous recombination to substitute the endogenous promoter of *CYM1*.

The activity can be used intracellularly to generate peptides that do not require post-translational modifications from the secretory pathway, such as disulfide bond formation, *N*- and *O*-glycosylation or exoprotease activity.

The role of Cym1p cytosolic activity in intracellular peptide synthesis is shown in the biosynthesis of CCK-22 in wild type cells compared to the isogenic strain with a *CYM1* disruption, which shows a significant increase in the amount of CCK-22 (Fig. 8C). Synthesis of the peptide of interest should be performed in such a way that translocation into the endoplasmatic reticulum (ER) is avoided. This can be performed either by removal of the hydrophobic amino-terminal signal sequence from proteins that enter the ER post-translationally, or by expression in a temperature sensitive secretory mutant such as *sec61*, which abolishes translocation of secretory peptides into the ER when the temperature is elevated to 37°C.

The propeptide or prepropeptide of interest will then be cytosolically located and a potential substrate for Cym1p. Release of the peptide from its precursor will be carried out by the Cym1p activity by introduction of the cleavage site seen from proCCK, which results in the release of Gly-extended CCK-22 after endoproteolytic processing C-



terminal to Lys<sup>61</sup> (Ser-Ile-Val-Lys<sup>61</sup> ↓) (Fig. 13A). If the peptide of interest is GLP1, synthesis can be performed as a fusion to a Cym1p cleavage site, which could be part of proCCK (Fig. 13B). The peptide of interest will then accumulate in the cytosol and can be purified from sedimented cells after lysis.

5

Expression of proBNP in *cym1* mutant strain enhance proBNP secretion

To elucidate the role of *CYM1* in the biosynthesis of proBNP *in vivo*, proBNP was expressed in the the vacuole deficient strain, BJ2168 and the three protease deficient  
10 isogenic strains,  $\Delta cym1::LEU2$  (LJY430),  $\Delta yps1::TRP1$  (LJY440) and a  $\Delta cym1::LEU2$   
 $\Delta yps1::TRP1$  (LJY431). Deletion of *CYM1* resulted in an approximately 100% increase in the total amount of secreted proBNP, whereas the proBNP secretion was independent on disruption of the gene encoding the aspartyl protease, Yps1p and was thereby  
15 similar to the wildtype strain (Fig. 15A). Disruption of both *Cym1* and *Yps1* was as expected similar to the secreted amount in a *cym1* mutant (Fig. 15A).

Analysis of the proBNP in secreted from a *cym1* mutant

To analyse the proBNP expressed in *Saccharomyces cerevisiae*, media from a *cym1*  
20 mutant was applied to FPLC chromatography and analysed by RIA using Ab. 98192. The peak eluting from fraction 34-39 corresponds to intact proBNP, whereas the peak eluting in fraction 53-62 is a processed form of proBNP, most likely the proBNP fragment 1-76 (Fig. 15B). The release of fragment 1-76 and BNP-32 from proBNP, is due to cleavage after a single Arg residue and is probably due to either Kex2 or Yps1  
25 activity.

## Discussion

The secreted polypeptides varies with the growth conditions, the fraction of CCK-22 increasing when the culture reaches stationary phase, while the intracellularly processed fraction remains unaltered under stress conditions. The increase in  
30 extracellular cleavage to CCK-22 as the cells enter stationary phase could indicate that extracellular endoproteases with the ability to process proCCK to CCK-22 are secreted or expressed on the cell membrane. It is known that the aspartyl proteases, Yps1p and Yps2p, exhibit cell surface activity (Komano et al., 1998). In addition, it has previously been shown that heterologous peptide expression in a *yps1* strain improved the  
35 recovery of proteins and peptides like albumin, glucagon, GLP1, GLP2 and CART by inhibiting proteolysis C-terminal to mono-basic residues (Egel-Mitani et al., 2000;

Kerry-Williams et al., 1998). Thus, recent studies ((Egel-Mitani et al., 2000; Kerry-Williams et al., 1998) and those of the present inventors) show the importance of collecting secreted peptides during exponential growth in order to avoid additional extracellular processing.

5

ProCCK expressed in a vacuole protease deficient strain showed 30% intracellular processing at Lys<sup>61</sup> in proCCK. The fraction of extracellular Lys<sup>61</sup>-processing is, however, decreased to 2/3 of the observed fraction within intact yeast cells, which reveals an intracellular degradation of CCK-22 prior to secretion. The increase in  
10 extracellular proteolysis under limited nutrient resources is probably due to an activation or upregulation in transcription of the extracellular proteases under limited nutrient resources as seen with the upregulation of *YPS1* transcription during stationary phase (Gasch et al., 2000). Part of the cell surface activity can be assigned to the yapsins, Yps1p, Yps2p and Yps3p, but some extracellular activity was sustained even in  
15 the triple mutant.

In the present study, the inventors have shown that deletion of *KEX2* causes a 5 fold reduction in both the intracellular and extracellular Lys<sup>61</sup>-cleavage. The *kex2* strain expressing proCCK do not only alter the cleavage of Lys<sup>61</sup> in proCCK, it also changes  
20 the intracellular retention time of CCK as the intracellular concentration of CCK peptides is reduced with more than 60%, while the extracellular CCK concentration is increased by almost 60% compared to wild type yeast. Moreover, analysis of the secreted CCK peptides from the *kex1 kex2* double mutant and the *kex2* mutant showed disappearance of the Tyr<sup>45</sup>-Val<sup>60</sup> degradation product. Thus, the removal of Lys<sup>61</sup> by  
25 Kex1p was abolished in a *kex2* strain indicating an enhanced secretion rate through the *trans*-Golgi network. These results and the observations on the rapid secretion of proCCK suggest that it may be the intracellular retention caused by Kex2p that leads to an increased synthesis of CCK-22 in wild type yeast by Yps1p and probably to some extent by Kex2p.

30

The type of protease responsible for the intracellular maturation of CCK-22 was investigated in an *in vitro* protease assay using a crude extract of *S. cerevisiae* to analyse the processing of synthetic human CCK-33 to CCK-22 in the presence of different inhibitors. By not including detergents in extraction of protease activity,  
35 activity from Kex2p as well as the GPI-anchored yapsins was avoided (Azaryan et al., 1993; Fuller et al., 1989; Komano et al., 1999). Of the inhibitors tested, the proteolysis was only inhibited by EDTA and 1,10 ortho-phenanthroline, and the activity could be

restored by addition of the divalent cations  $Zn^{2+}$ ,  $Co^{2+}$  and  $Mn^{2+}$ . This indicated that a metalloprotease participates in the maturation of CCK-22.

None of the candidate metalloproteases contain an obvious signal peptide to direct the  
5 protein into ER. Therefore, the inventors investigated strains deficient in each of the  
metalloproteases with the exception of mitochondrial proteases. Expression of proCCK  
in each of the strains resulted in unaltered maturation of CCK-22 similar to that seen in  
wild type yeast. However, by using the *in vitro* protease assay the inventors identified  
Cym1p as an endoprotease performing post-Lys cleavage of CCK-33. That Cym1p can  
10 cleave Lys<sup>61</sup> in proCCK was verified by overexpression studies, showing a several fold  
increase in enzyme activity.

Intracellular synthesis of CCK-22 was decreased in a *cym1* strain accompanied by an  
increased concentration of total proCCK. In contrast, the fraction of extracellular  
15 CCK-22 was increased compared to wild type yeast with a parallel increase in total  
CCK. These findings are in accordance with a cytosolic location of the Cym1p activity  
like most insulin-degrading enzymes (Bai et al., 1996) and show that it acts on the  
preproMf $\alpha$ 1p-proCCK construct prior to translocation into the endoplasmatic reticulum.  
Thus, the pre-translocational degradation of proCCK is decreased by *CYM1* disruption  
20 and the total production increased.

Expression of proBNP as a fusionpeptide to the preproMf $\alpha$ 1p sequence in a *cym1 $\Delta$*   
mutant shows a two-fold increase of the extracellular proBNP content compared to the  
wild type strain. Analysis of the secreted proBNP by chromatography disclosed that two  
25 major forms were present. One is the entire proBNP, whereas the other is the proBNP  
fragment 1-76, thus the biologically active BNP-32 is also synthesised, though not  
detectable in the present assay. The release of proBNP fragment 1-76, most likely  
depends on the Kex2p activity, however this could not be tested in the present assay,  
since the release of proBNP depends on both Kex2p and Kex1p.

30

All publications discussed above are incorporated herein in their entirety.

It will be appreciated by persons skilled in the art that numerous variations and/or  
modifications may be made to the invention as shown in the specific embodiments  
35 without departing from the spirit or scope of the invention as broadly described. The  
present embodiments are, therefore, to be considered in all respects as illustrative and  
not restrictive.

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**Claims**

1. A method for producing a protein of interest in a host cell, wherein said host cell has been genetically modified in order to express significantly reduced levels of a metalloprotease comprising a HXXEH motif (SEQ ID NO 1), compared to the corresponding  
5 non-modified cell when cultured under identical conditions, the method comprising
  - a) introducing into the host cell a nucleic acid sequence encoding the protein of interest,
  - 10 b) cultivating the host cell of step (a) in a suitable growth medium for production of the protein of interest, and
  - c) isolating the protein of interest.
- 15 2. A method according to claim 1, wherein the metalloprotease further comprises a glutamic acid residue between 70 and 80 amino acids C-terminal of the second His residue in the HXXEH motif.
- 20 3. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a glycine residue 3 amino acids N-terminal of the first His residue in the HXXEH motif.
- 25 4. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a glycine residue 5 amino acids C-terminal of the second His residue in the HXXEH motif.
- 30 5. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a lysine residue 8 amino acids C-terminal of the second His residue in the HXXEH motif.
6. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a tyrosine residue 9 amino acids C-terminal of the second His residue in the HXXEH motif.
- 35 7. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a proline residue 10 amino acids C-terminal of the second His residue in the HXXEH motif.

8. A method according to any of the preceeding claims, wherein the metalloprotease further comprises the consensus sequence SEQ ID NO 2.
9. A method according to any of the preceeding claims wherein the metalloprotease further  
5 comprises the consensus sequence SEQ ID NO 3.
10. A method according to any of the preceeding claims, wherein the metalloprotease further comprises a NAXTXXXXT motif between 20 and 30 amino acids C-terminal of the second His residue in the HXXEH motif.
- 10 11. A method according to any of the preceeding claims, wherein the metalloprotease is selected from:
- i) any one of the group consisting of SEQ ID NO's 4 to 15, and  
15 ii) a sequence which is at least 80% identical to any one of SEQ ID NO's 4 to 15.
12. A method according to any of the preceeding claims, wherein the metalloprotease is at least 80% identical to the SEQ ID NO: 4.
- 20 13. A method according to any of the preceeding claims, wherein the total amount of the protein of interest is increased at least 5% compared the corresponding non-modified cell when cultured under identical conditions.
- 25 14. A method according to any of the preceeding claims, wherein the total amount of the protein of interest is increased at least 50% more than the corresponding non-modified cell when cultured under identical conditions.
15. The method according to any of the preceeding claims, in which the host cell is a  
30 prokaryotic cell.
16. The method according to any of claims 1-14, in which the host cell is a eukaryotic cell.
17. The method according to claim 16, in which the host cell is a non-filamentous fungal  
35 cell.
18. The method according to claim 16, in which the host cell is a filamentous fungal cell.
19. The method according to claim 17, in which the host cell is a strain of *Saccharomyces*.



20. The method according to claim 19, in which the host cell is *Saccharomyces cerevisiae*.

21. A host cell useful for the expression of a protein of interest, wherein said cell has been  
5 genetically modified in order to express significantly reduced levels of a metalloprotease  
comprising a HXXEH motif (SEQ ID NO 1) than the corresponding non-modified cell when  
cultured under identical conditions.

22. A host cell according to claim 21, wherein the metalloprotease further comprises the  
10 consensus sequence SEQ ID NO 3.

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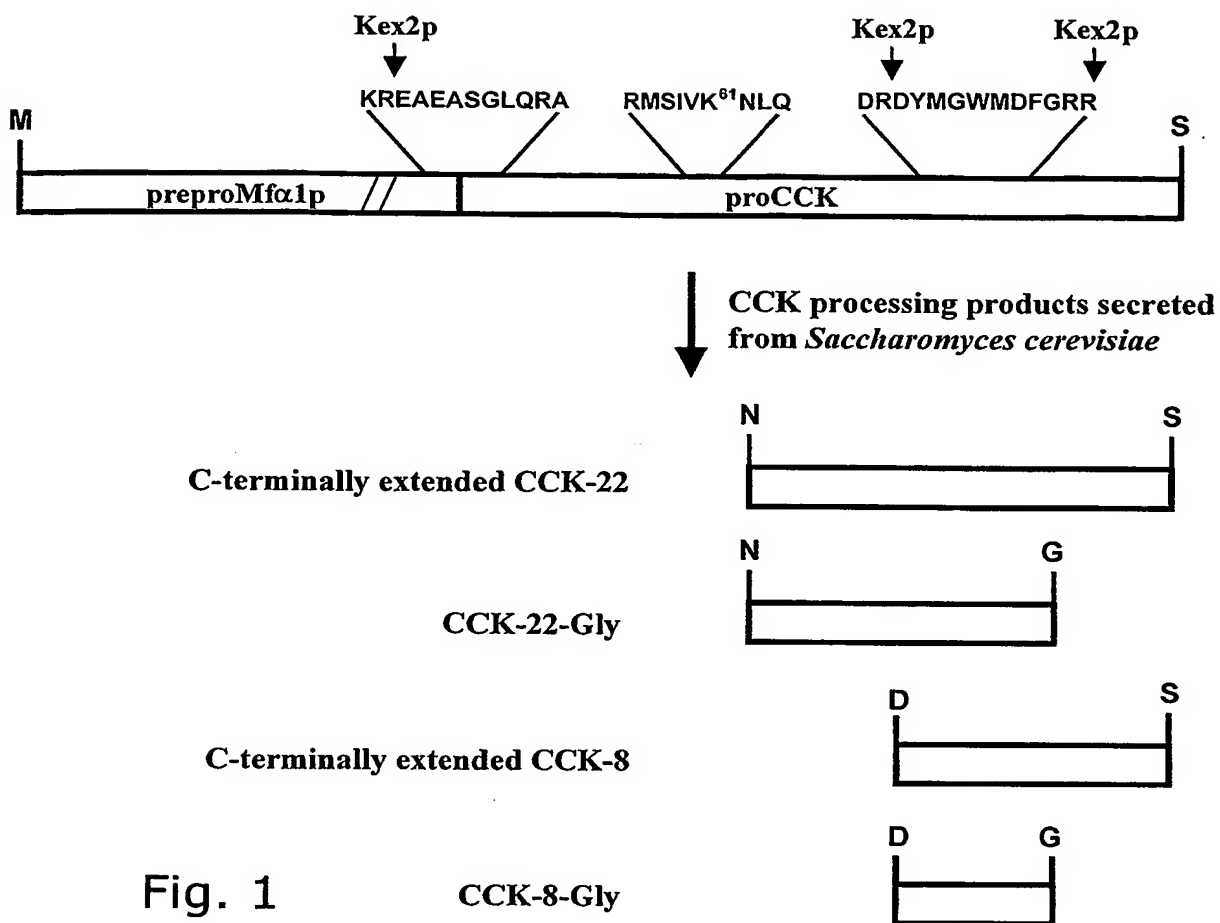


Fig. 1

CCK-8-Gly

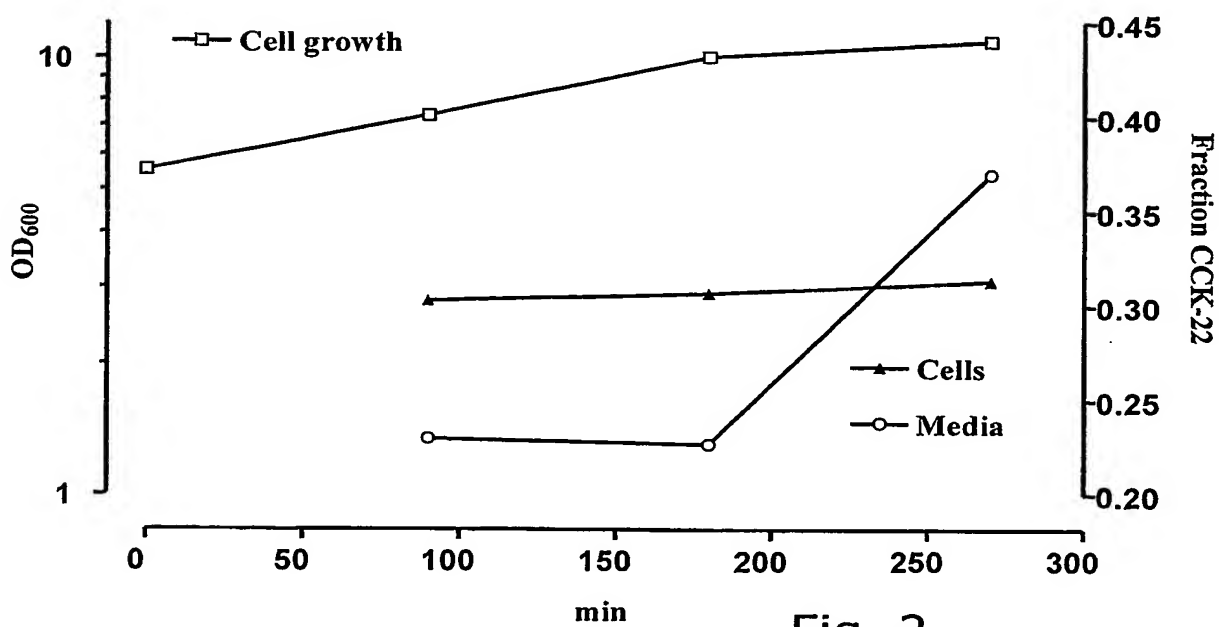


Fig. 2

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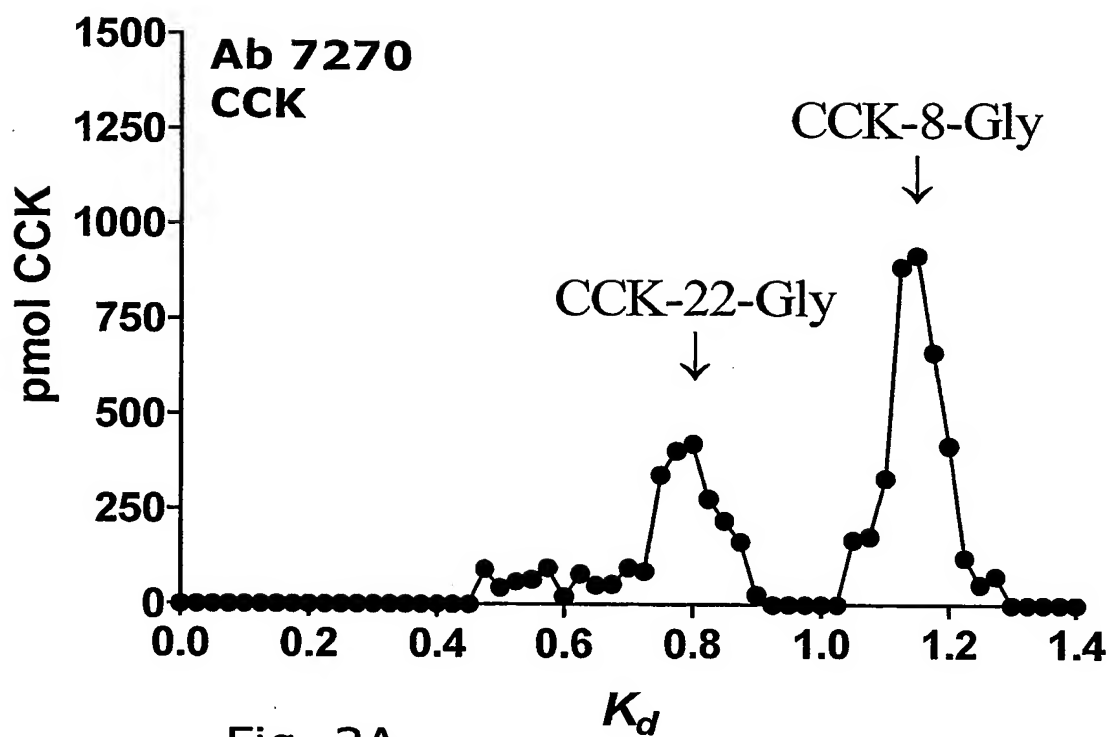


Fig. 3A

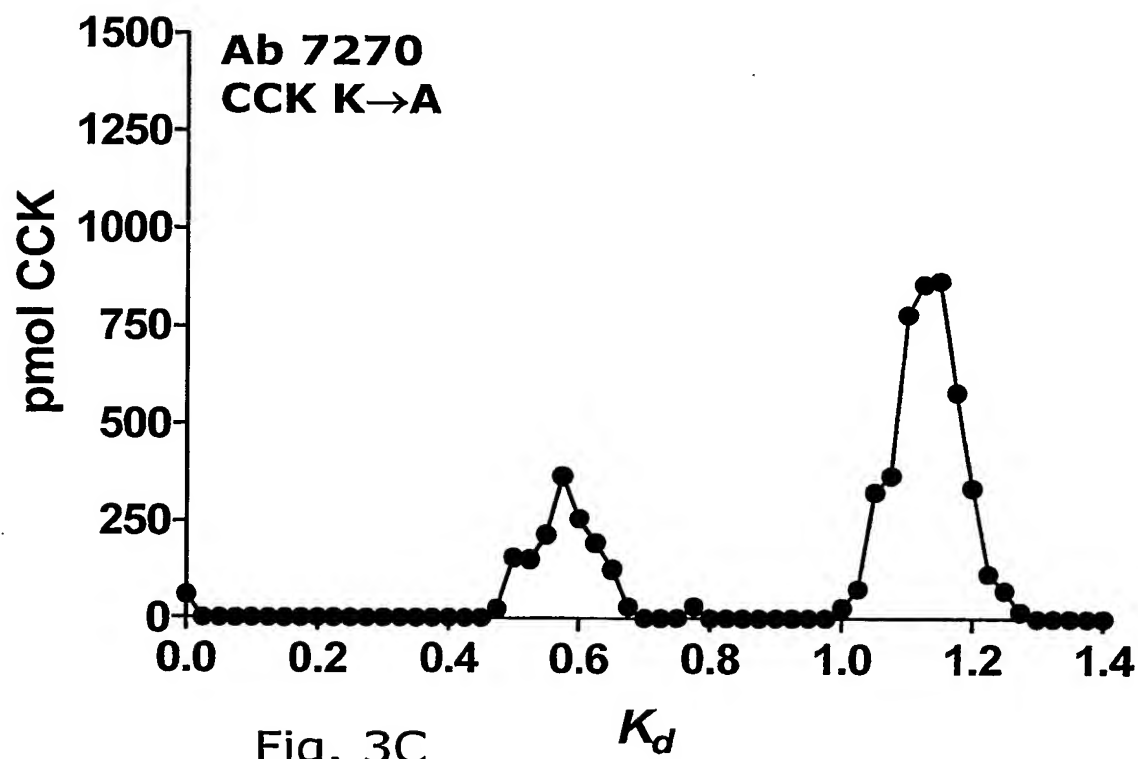
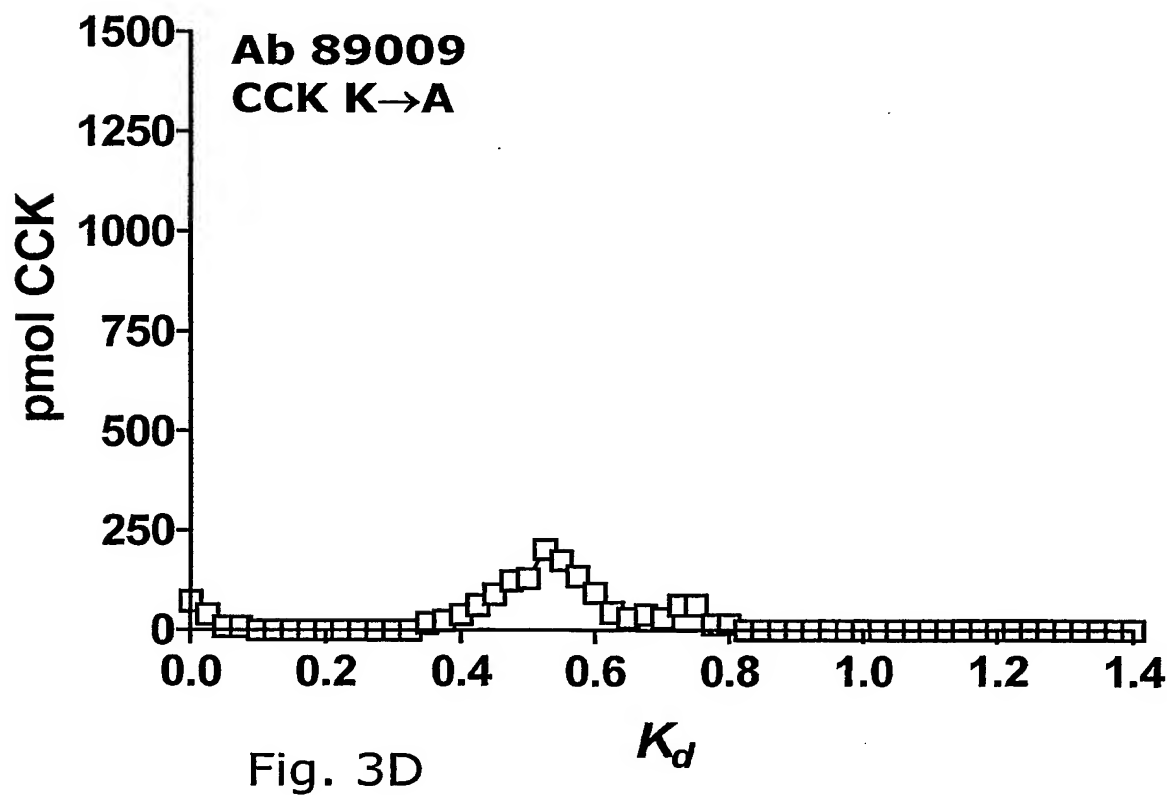
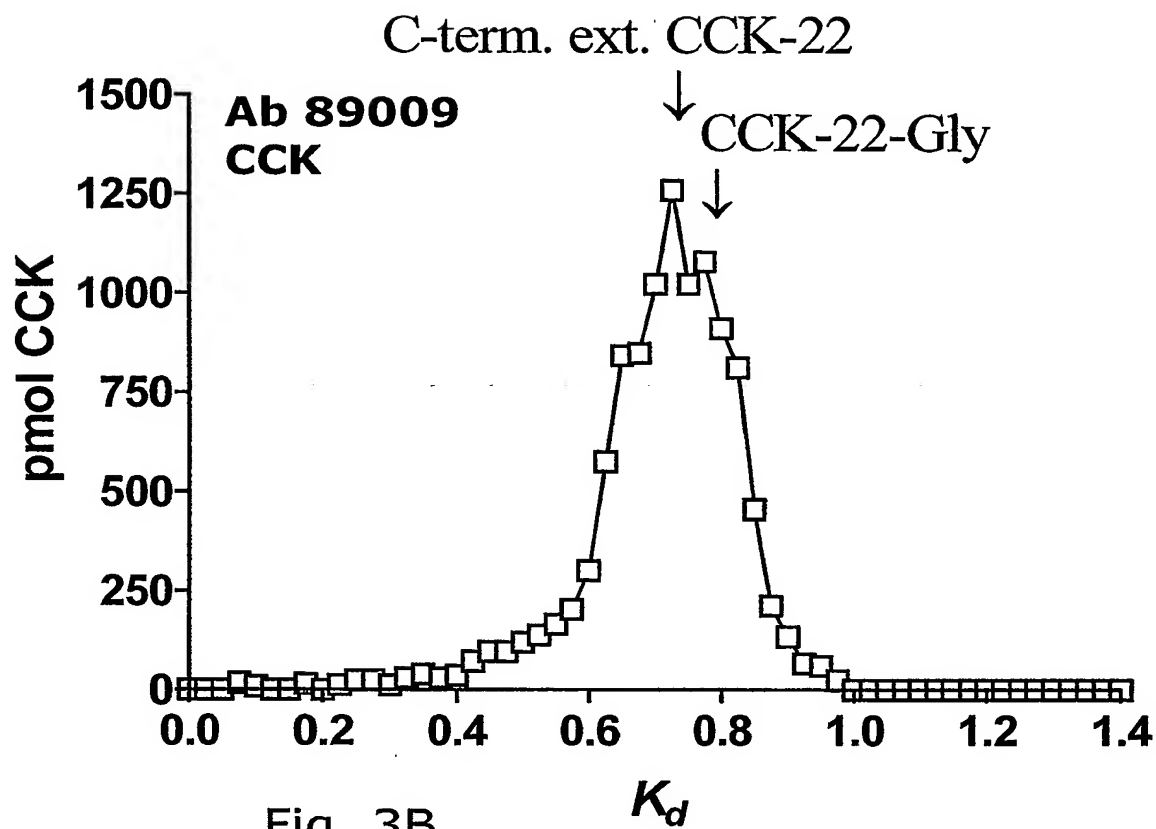
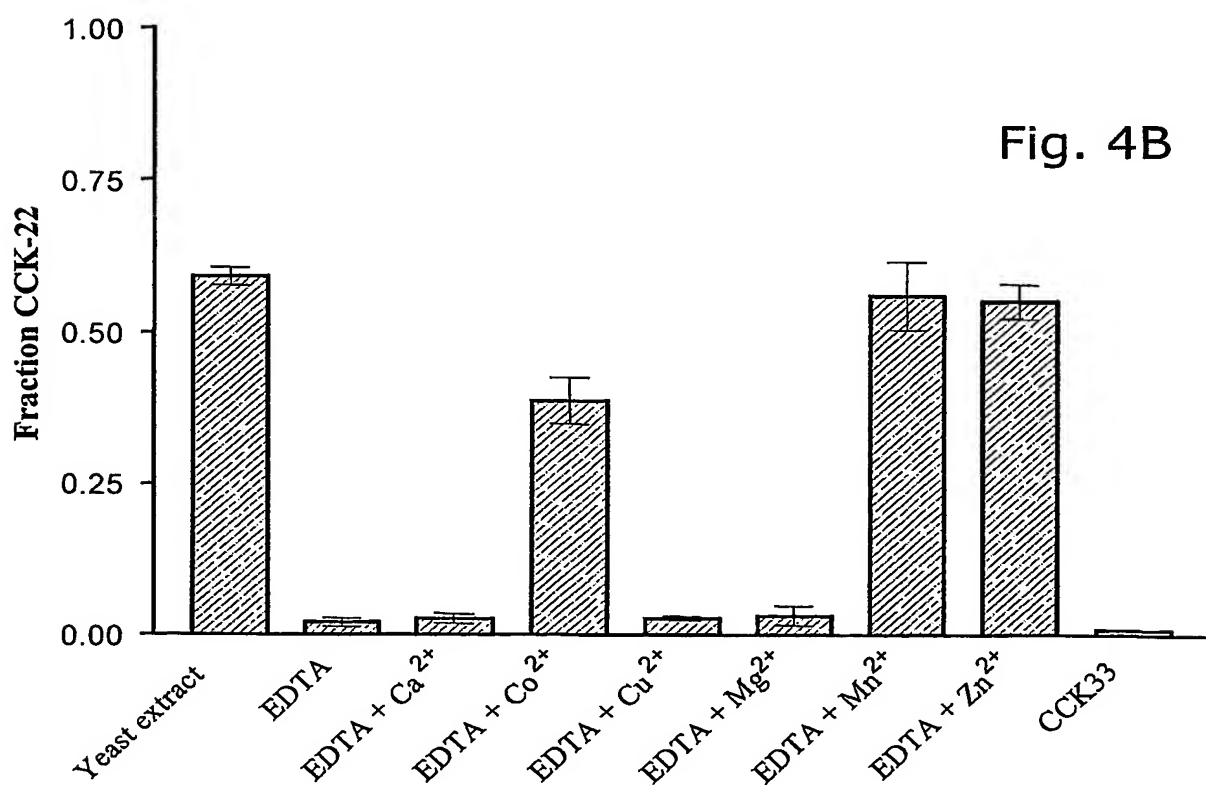
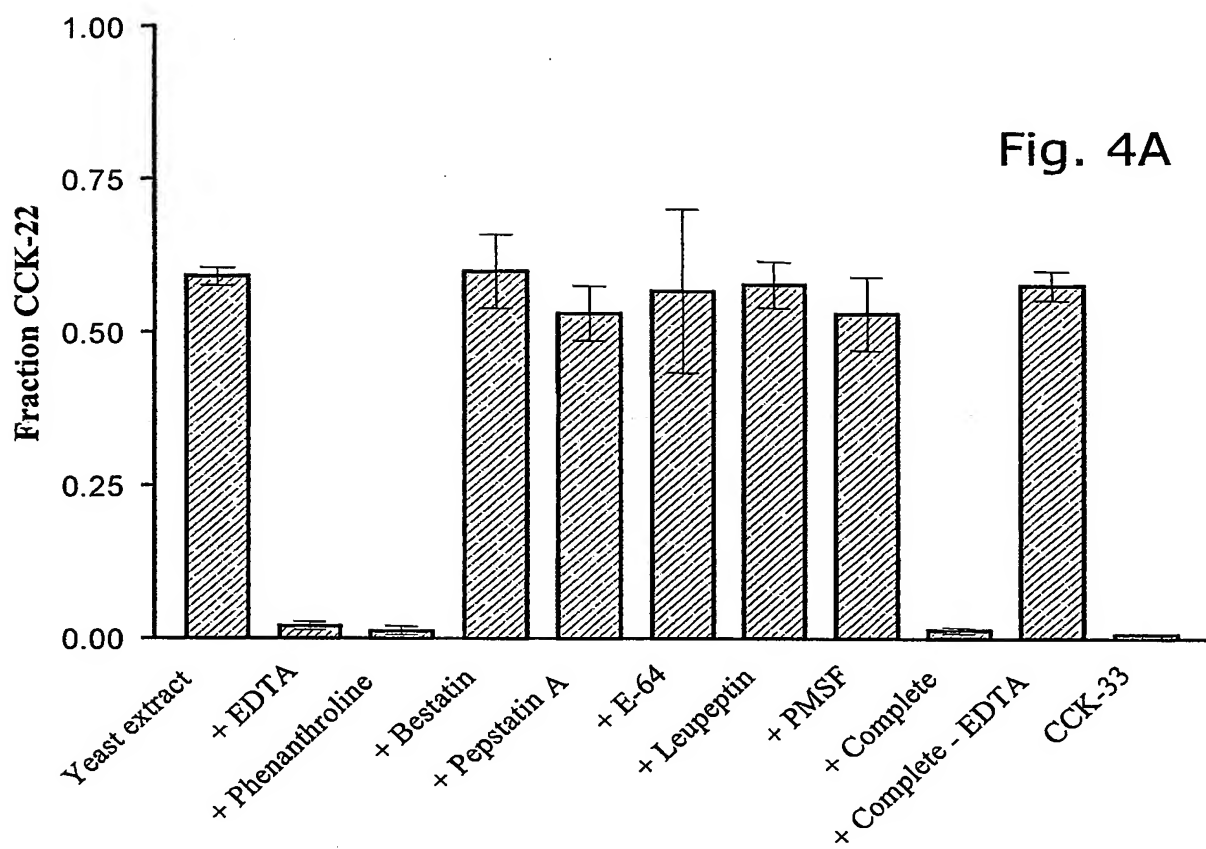


Fig. 3C

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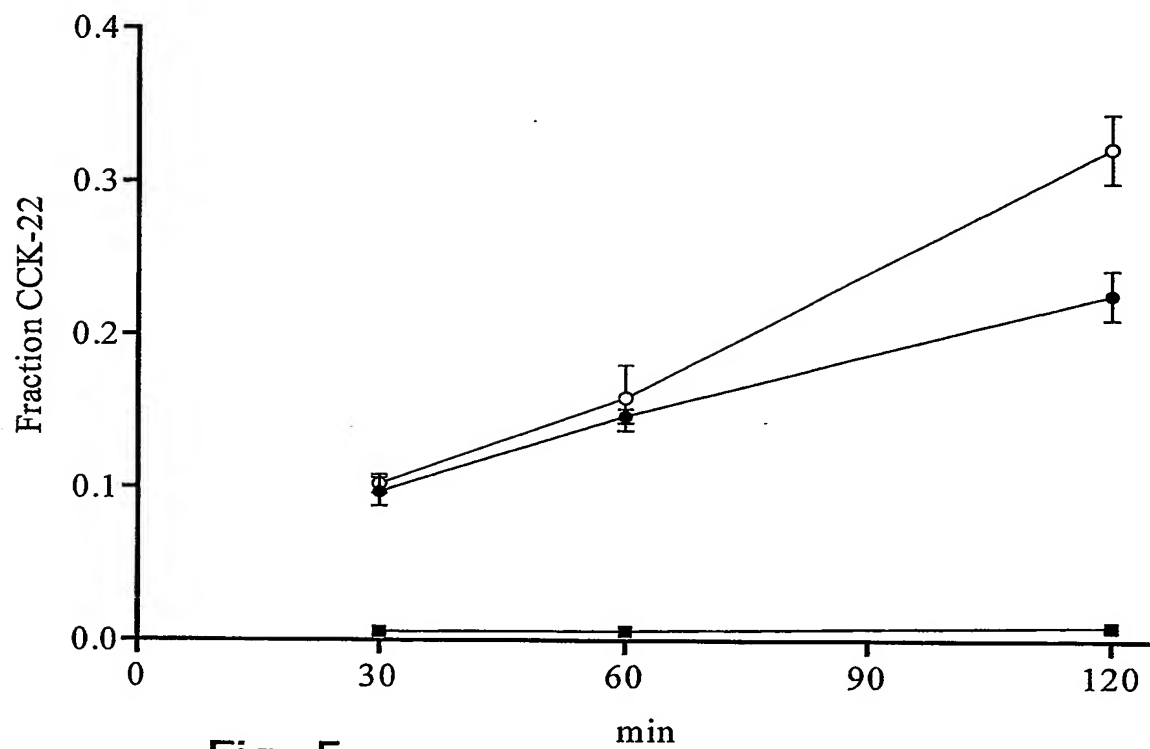


Fig. 5

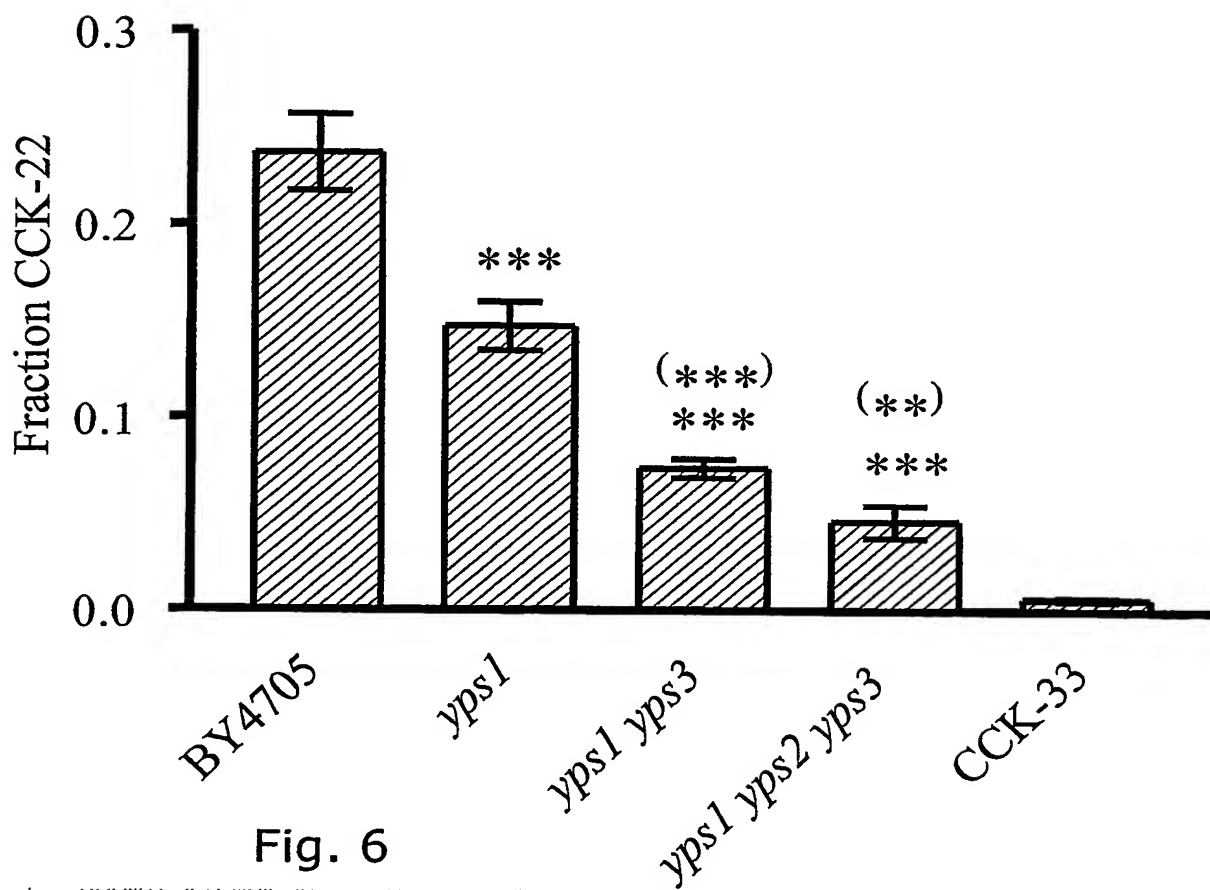
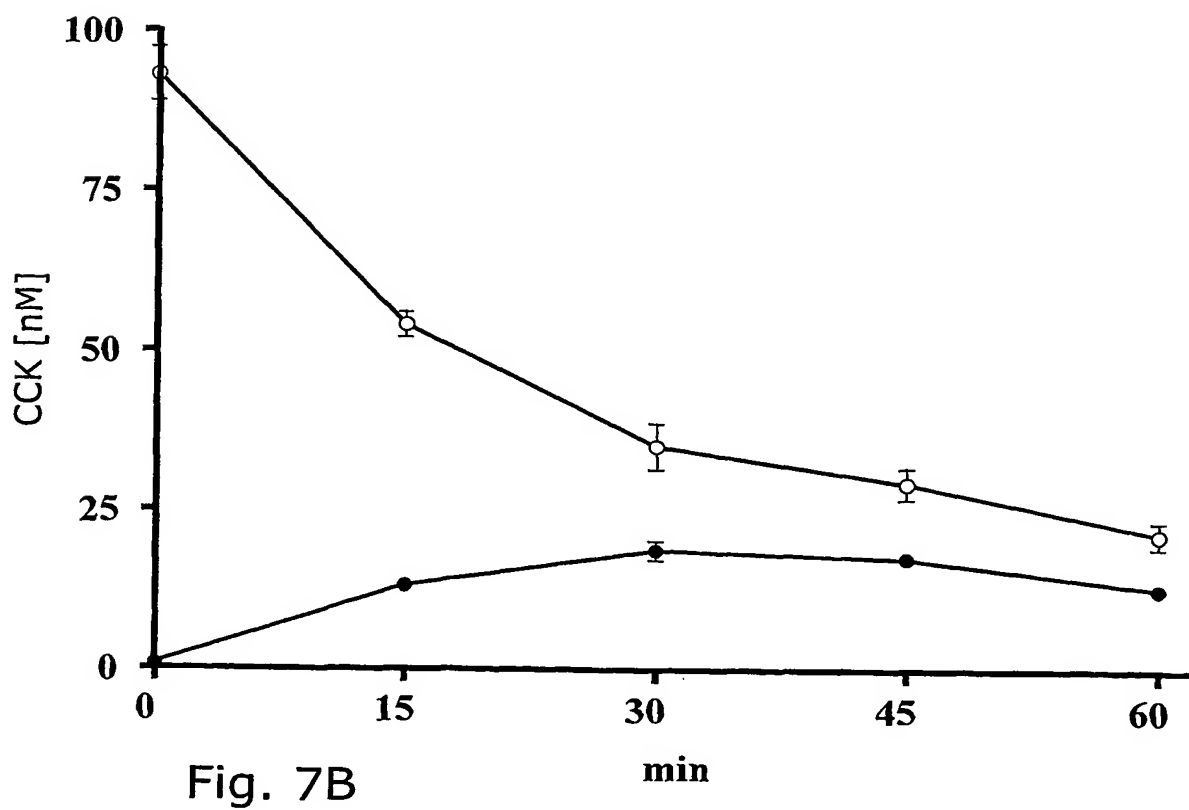
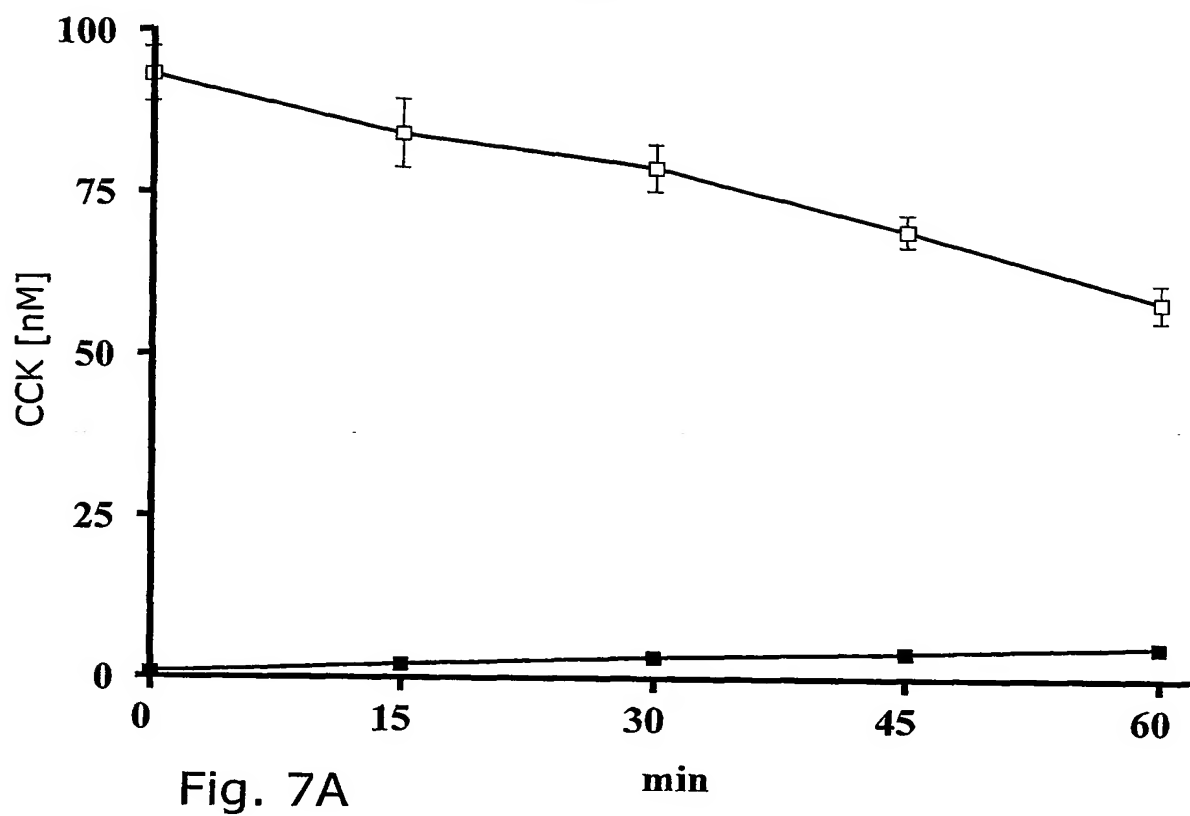


Fig. 6

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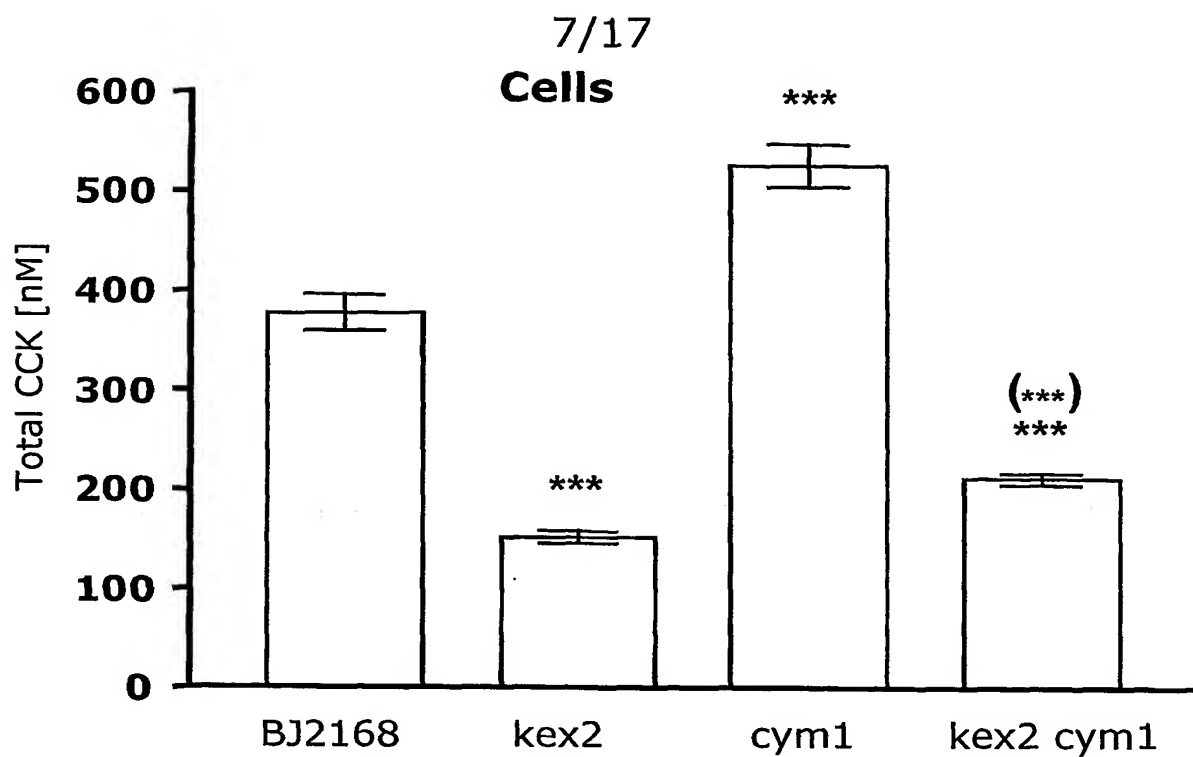


Fig. 8A

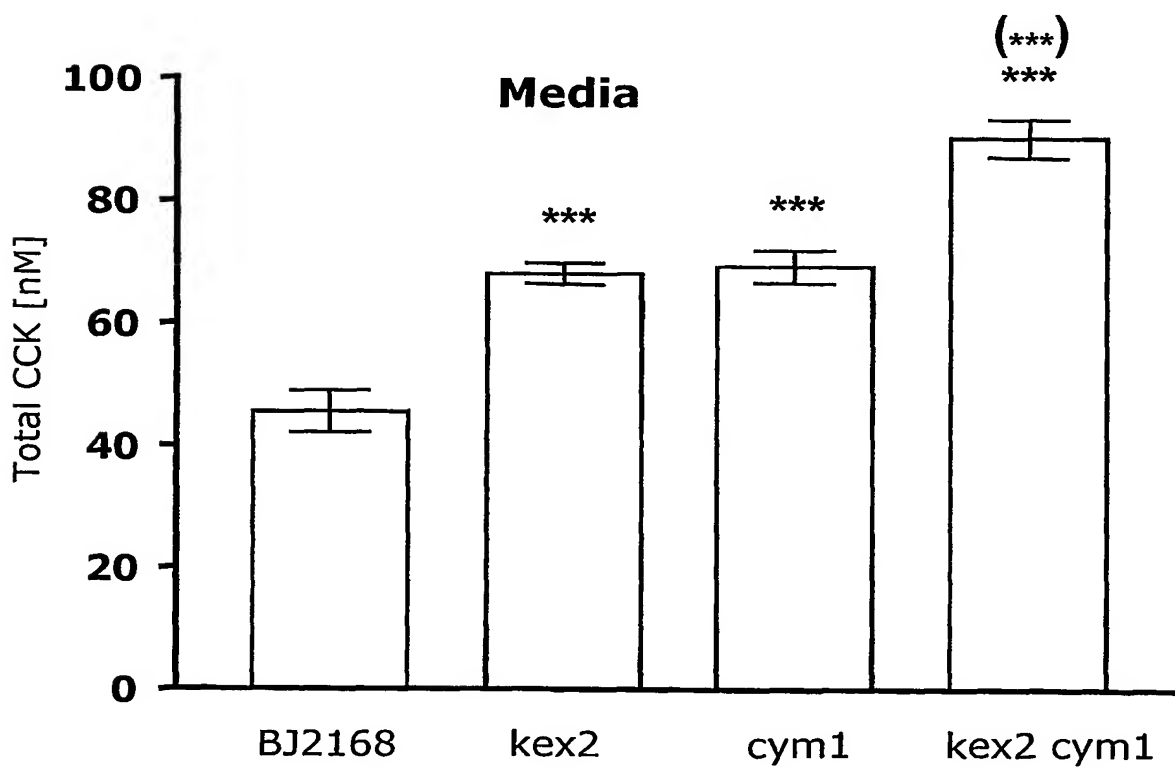


Fig. 8B



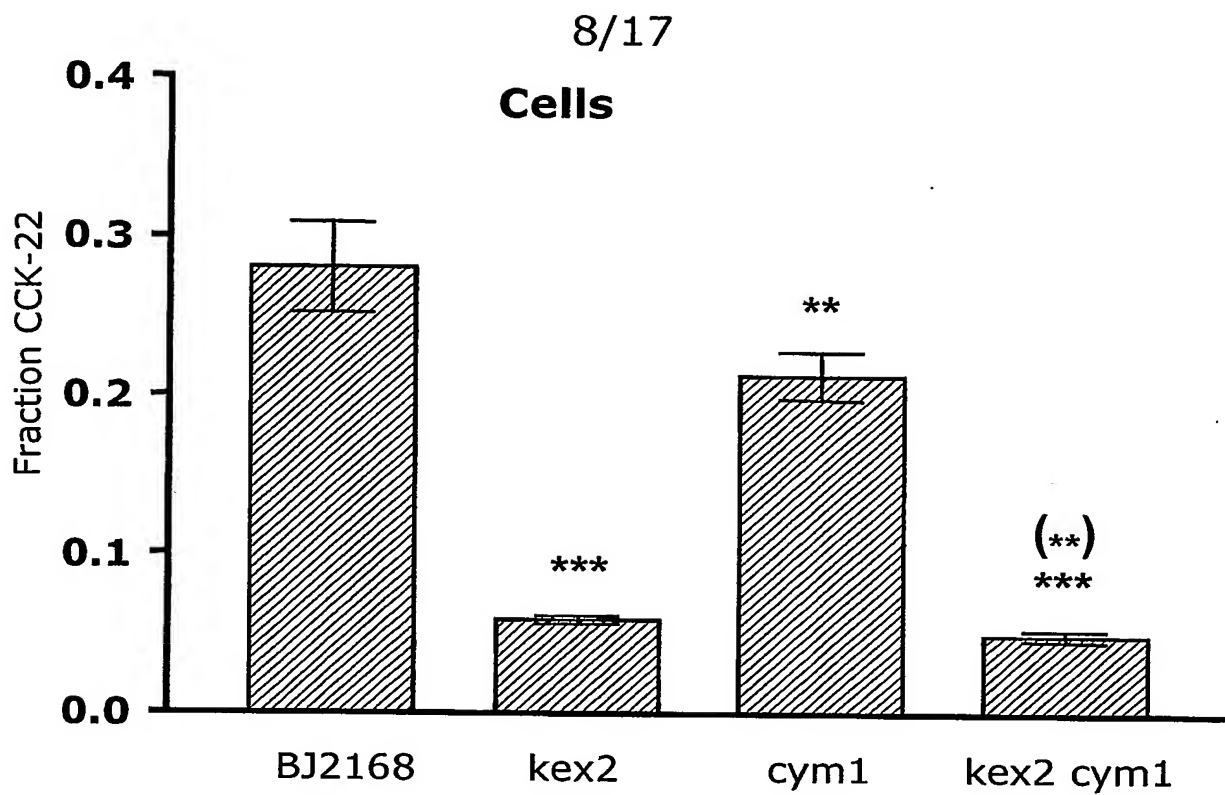


Fig. 8C

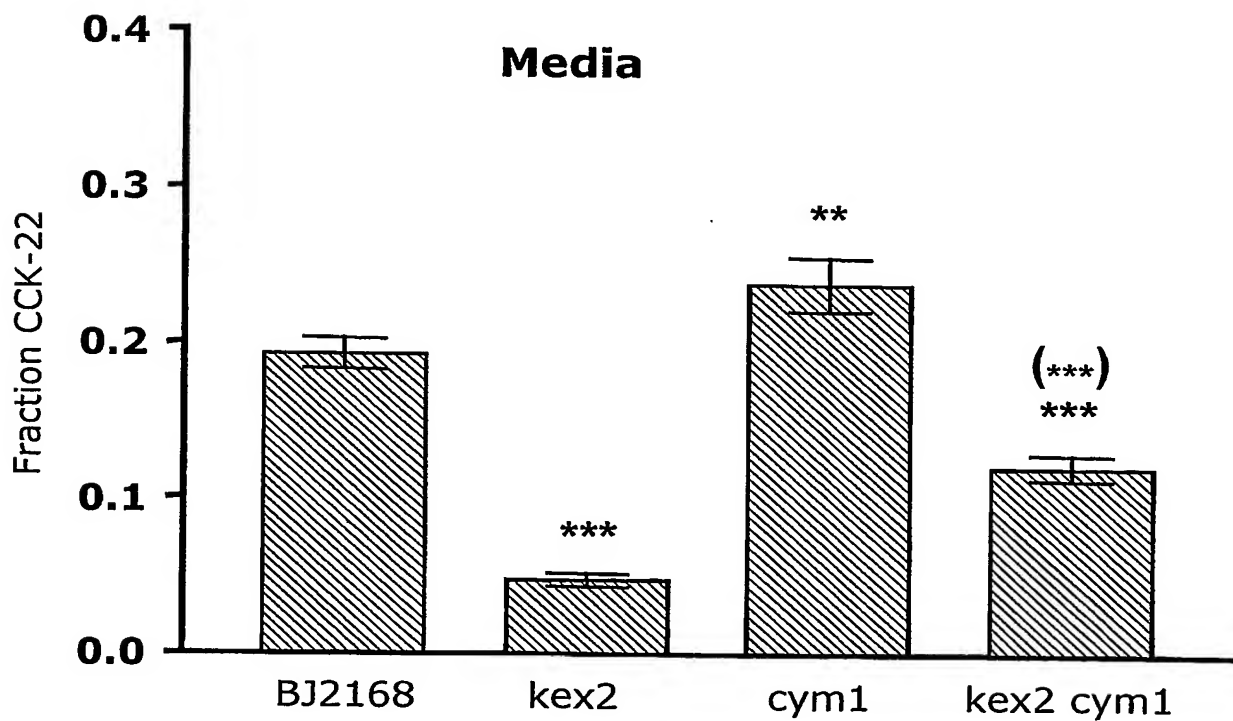


Fig. 8D

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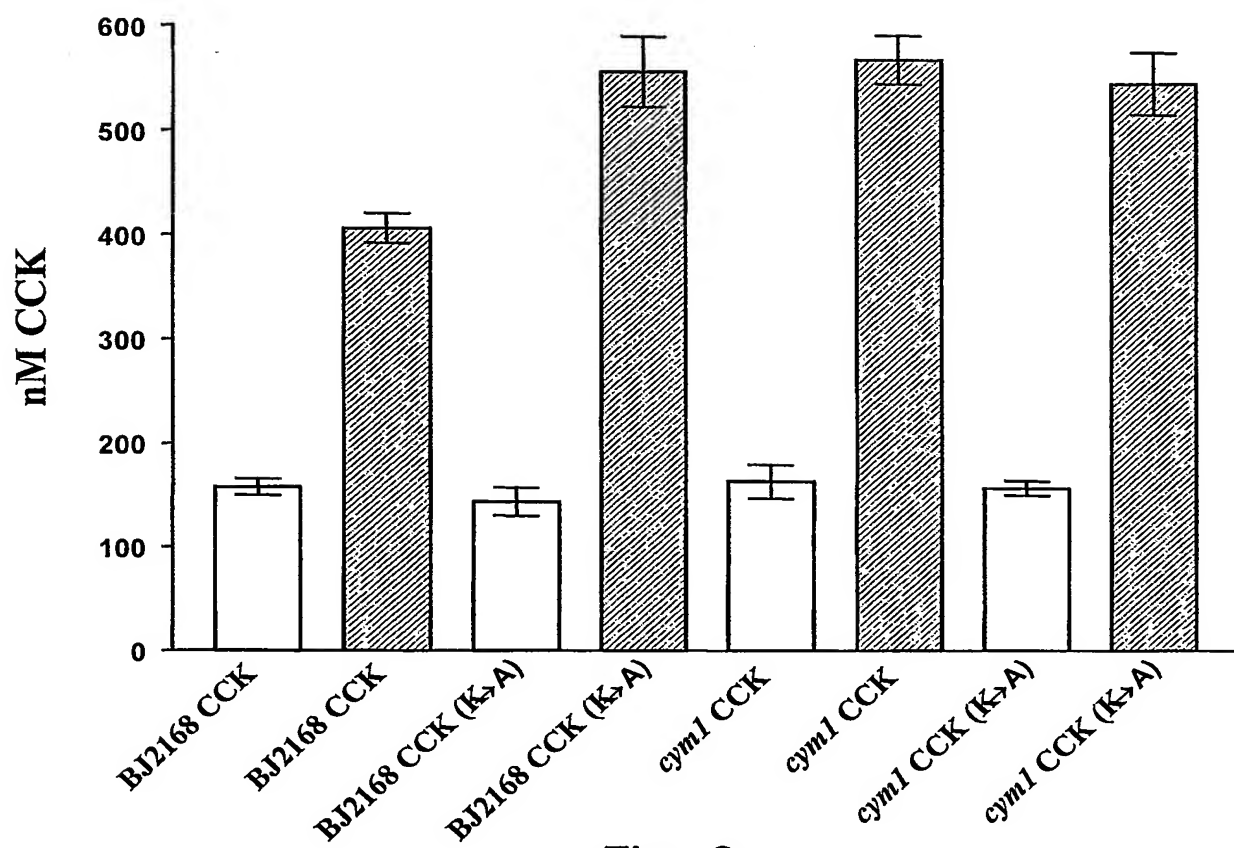
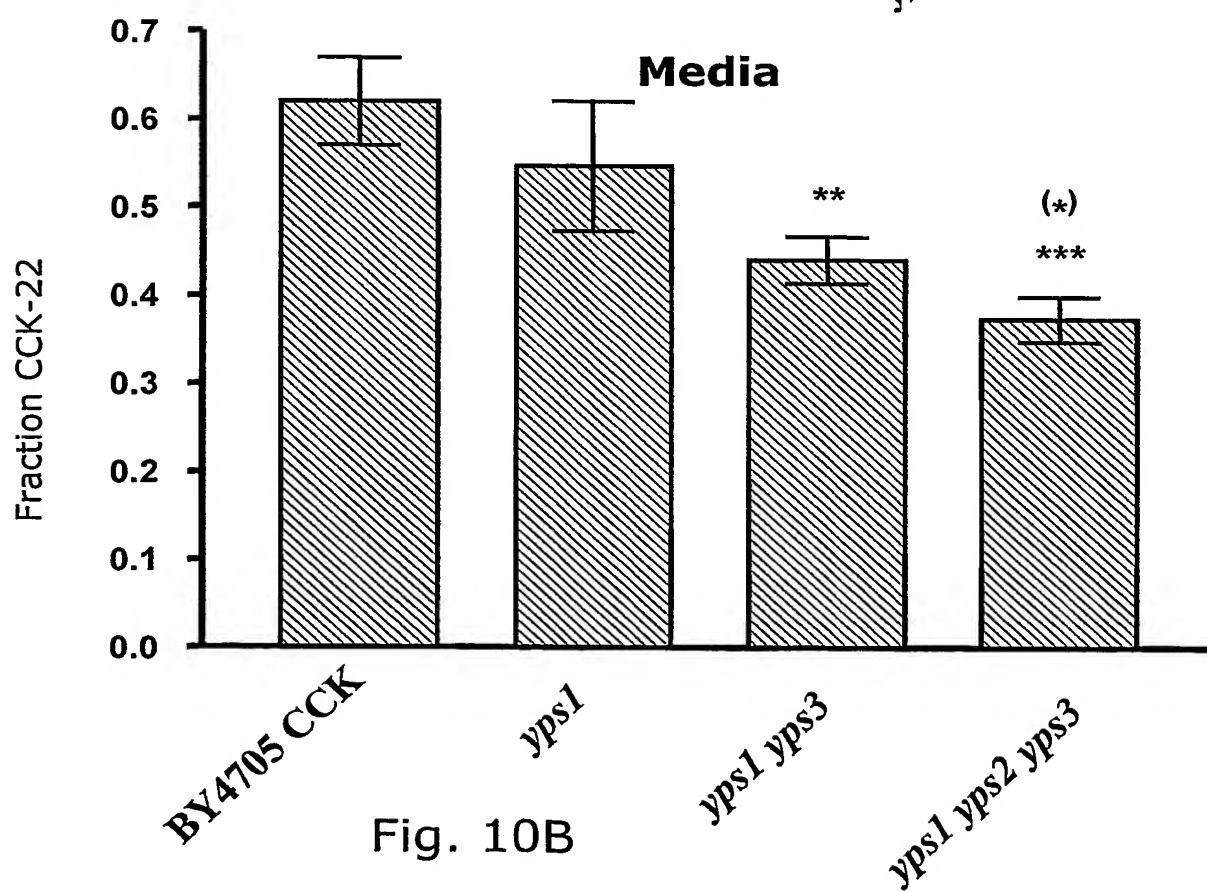
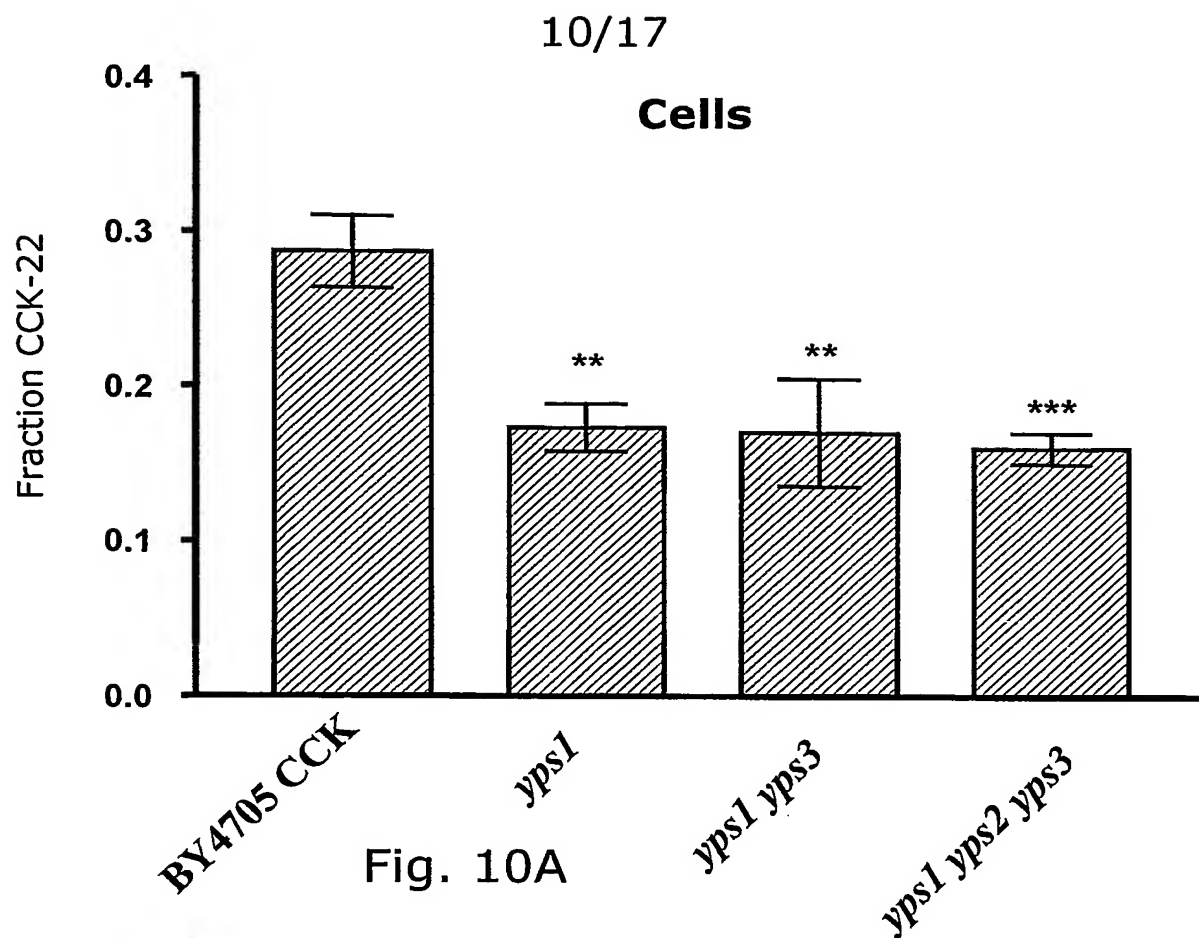
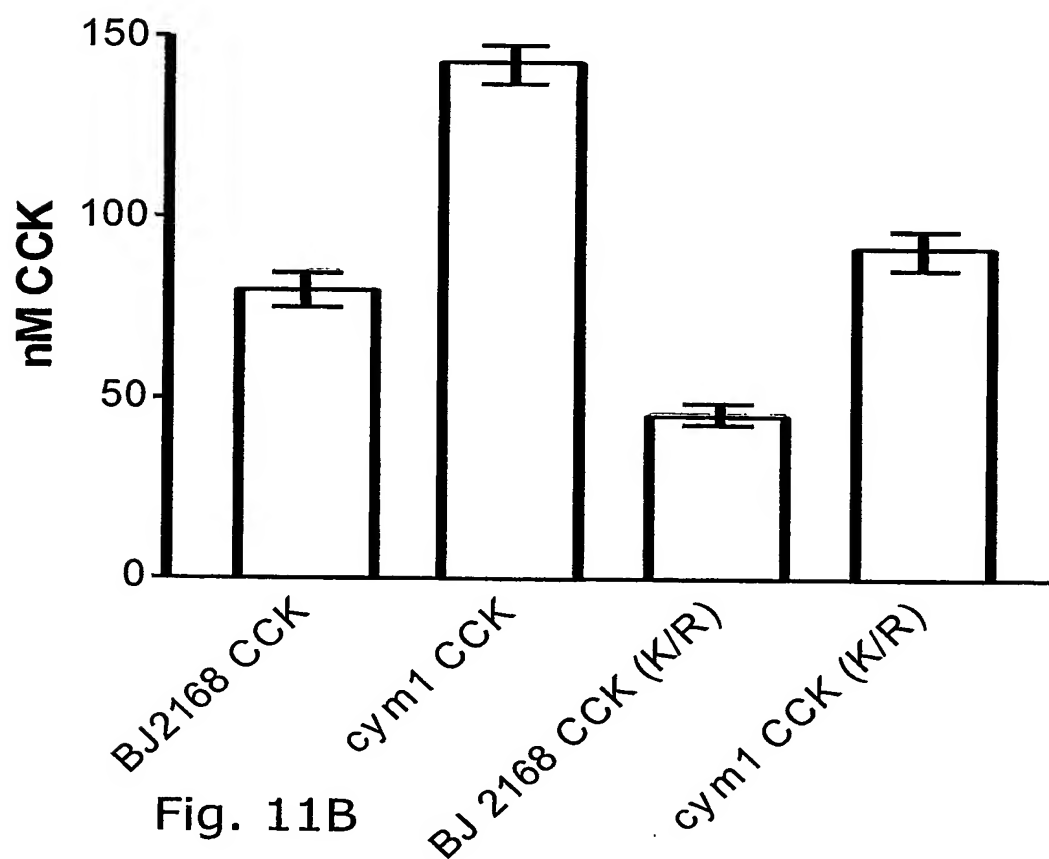
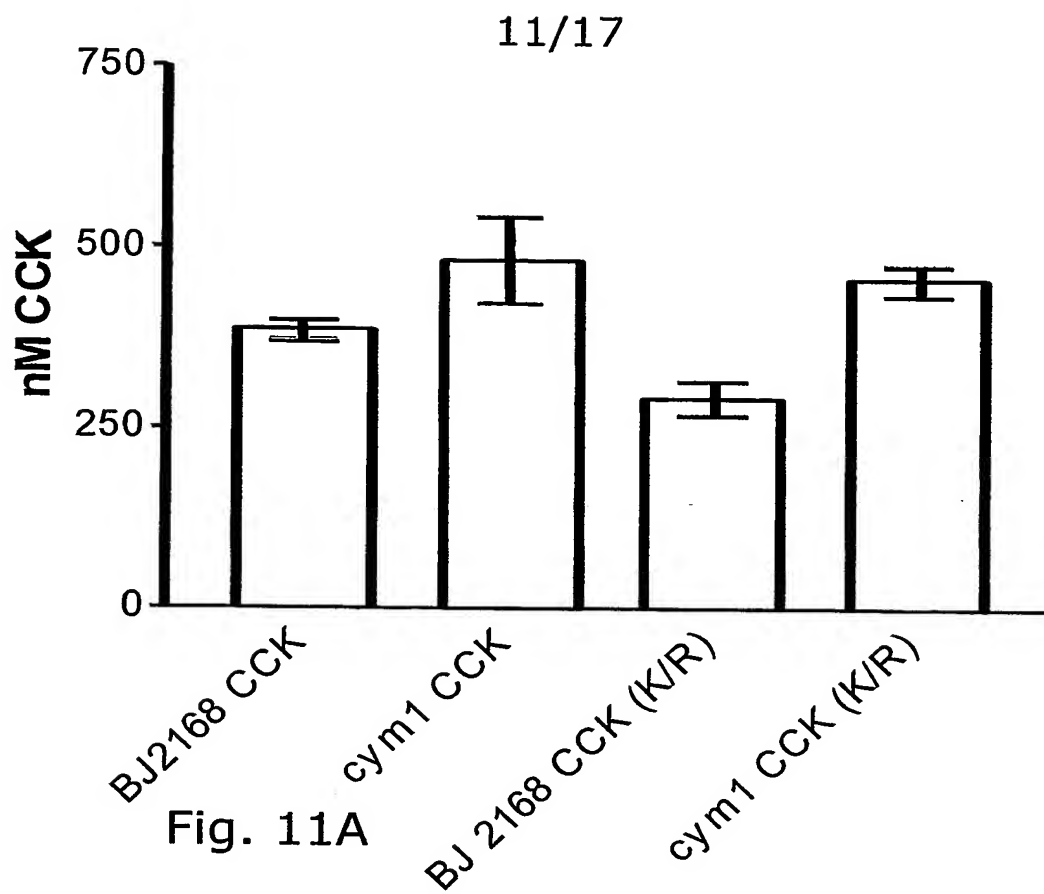


Fig. 9





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ProCCK				Molecular mass	Strain	SEQ ID NO:
PreproMfalp // .SGLQRAEEAPRRQLRV	CCK-61	CCK-58	CCK-39	CCK-22	CCK-8	43

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Fig. 12

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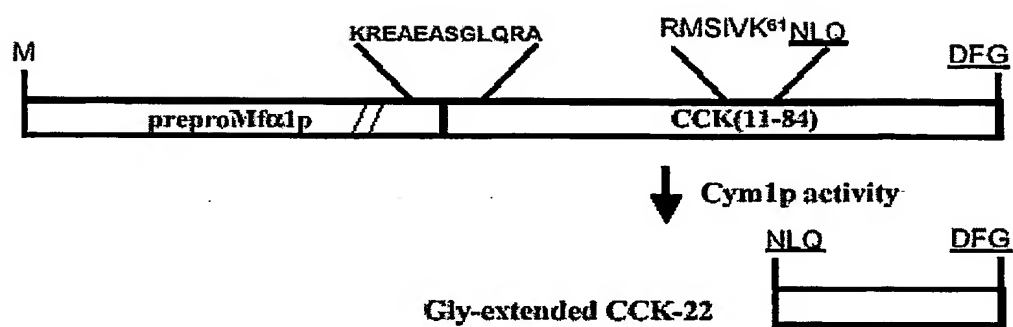


Fig. 13A

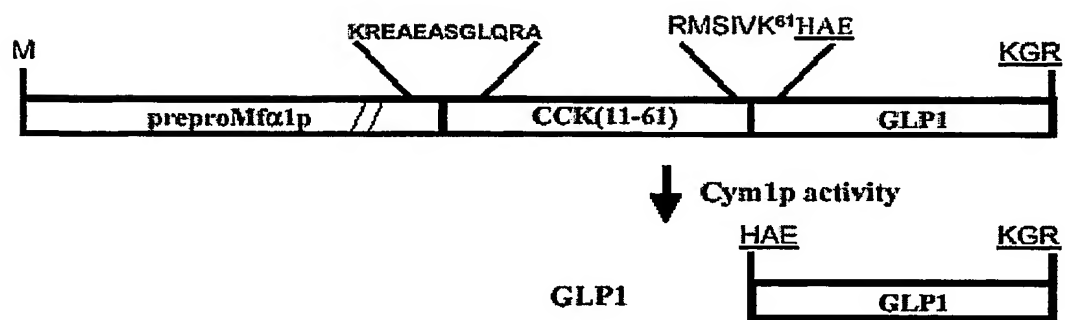


Fig. 13B

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M R F P S I F T A V L F A A S S A L A A -  
CCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGT  
61 -----+-----+-----+-----+-----+ 120  
GGTCAGTTGTGATGTTGTCTTCTACTTTGCCGTGTTTAAGGCCGACTTCGACAGTAGCCA  
P V N T T T E D E T A Q I P A E A V I G -  
TACTTAGATTTAGAAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAAT  
121 -----+-----+-----+-----+-----+ 180  
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TCTTTGGATAAAAGAGAGGCTGAAGCTCACCCGCTGGGCAGCCCCGGTTCAGCCTCGGAC  
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TTGGAAACGTCCGGGTTACAGGAGCAGCGCAACCATTTGCAGGGCAAACGTGTCGGAGCTG  
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Q V E Q T S L E P L Q E S P R P T G V W -  
AAGTCCCGGGAGGTAGCCACCGAGGGCATCCGTGGGCACCGCAAAATGGTCTCTACACC  
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K S R E V A T E G I R G H R K M V L Y T -  
CTGCGGGCACCACGAAGCCCCAAGATGGTGCAAGGGTCTGGCTGCTTTGGGAGGAAGATG  
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GACGCCCGTGGTGCTTCGGGGTTCTACCAGTTCCCAGACCGACGAAACCCTCCTTCTAC  
L R A P R S P K M V Q G S G C F G R K M -  
GACCGGATCAGCTCCTCCAGTGGCCTGGGCTGCAAAGTGCTGAGGCGGCATTAA  
541 -----+-----+-----+-----+-----+ 594  
CTGGCCTAGTCGAGGAGGTCACCGGACCCGACGTTTTCAGACTCCGCCGTAATT  
D R I S S S S G L G C K V L R R H \* -

Fig. 14A

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ATGAGATTTCTTCAATTTTACTGCAGTTTATTTCGCAGCATCCTCCGCATTAGCTGCT  
1 -----+-----+-----+-----+-----+-----+ 60  
TACTCTAAAGGAAGTTAAAAATGACGTCAAAATAAGCGTCGTAGGAGGCGTAATCGACGA  
M R F P S I F T A V L F A A S S A L A A -  
CCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCGGCTGAAGCTGTCATCGGT  
61 -----+-----+-----+-----+-----+ 120  
GGTCAGTTGTGATGTTGTCTTCTACTTTGCCGTGTTTAAGGCCGACTTCGACAGTAGCCA  
P V N T T T E D E T A Q I P A E A V I G -  
TACTTAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAAT  
121 -----+-----+-----+-----+-----+ 180  
ATGAATCTAAATCTTCCCCTAAAGCTACAACGACAAAACGGTAAAGGTTGTCGTGTTTA  
Y L D L E G D F D V A V L P F S N S T N -  
AACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTA  
181 -----+-----+-----+-----+-----+ 240  
TTGCCCAATAACAAATATTTATGATGATAACGGTCGTAACGACGATTTCTTCTTCCCCAT  
N G L L F I N T T I A S I A A K E E G V -  
TCTTTGGATAAAAGAGAGGCTGAAGCTAGCCCCAAGATGGTGCAAGGGTCTGGCTGCTTT  
241 -----+-----+-----+-----+-----+ 300  
AGAAACCTATTTTCTCTCCGACTTCGATCGGGGTTCTACCACGTTCACGACCGACGAAA  
S L D K R E A E A S P K M V Q G S G C F -  
GGGAGGAAGATGGACCGGATCAGCTCCTCCAGTGGCCTGGGCTGCAAAGTGCTGAGGCGG  
301 -----+-----+-----+-----+-----+ 360  
CCCTCCTTCTACCTGGCCTAGTCGAGGAGGTCACCGGACCCGACGTTTCACGACTCCGCC  
G R K M D R I S S S S G L G C K V L R R -  
CATTAA  
361 ----- 366  
GTAATT  
H \* -

Fig. 14B



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ATGAGATTTCTTCAATTTTTACTGCAGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCT  
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TACTCTAAAGGAAGTTAAAAATGACGTCAAATAAGCGTCGTAGGAGGCGTAATCGACGA  
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P V N T T T E D E T A Q I P A E A V I G -  
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121 -----+-----+-----+-----+-----+ 180  
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AACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTA  
181 -----+-----+-----+-----+-----+ 240  
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N G L L F I N T T I A S I A A K E E G V -  
TCTTTGGATAAAAAGAAGCCCCAAGATGGTGCAAGGGTCTGGCTGCTTTGGGAGGAAGATG  
241 -----+-----+-----+-----+-----+ 300  
AGAAACCTATTTTCTTCGGGGTTCTACCACGTTCCAGACCGACGAAACCCTCCTTCTAC  
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301 -----+-----+-----+-----+-----+ 354  
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Fig. 14C

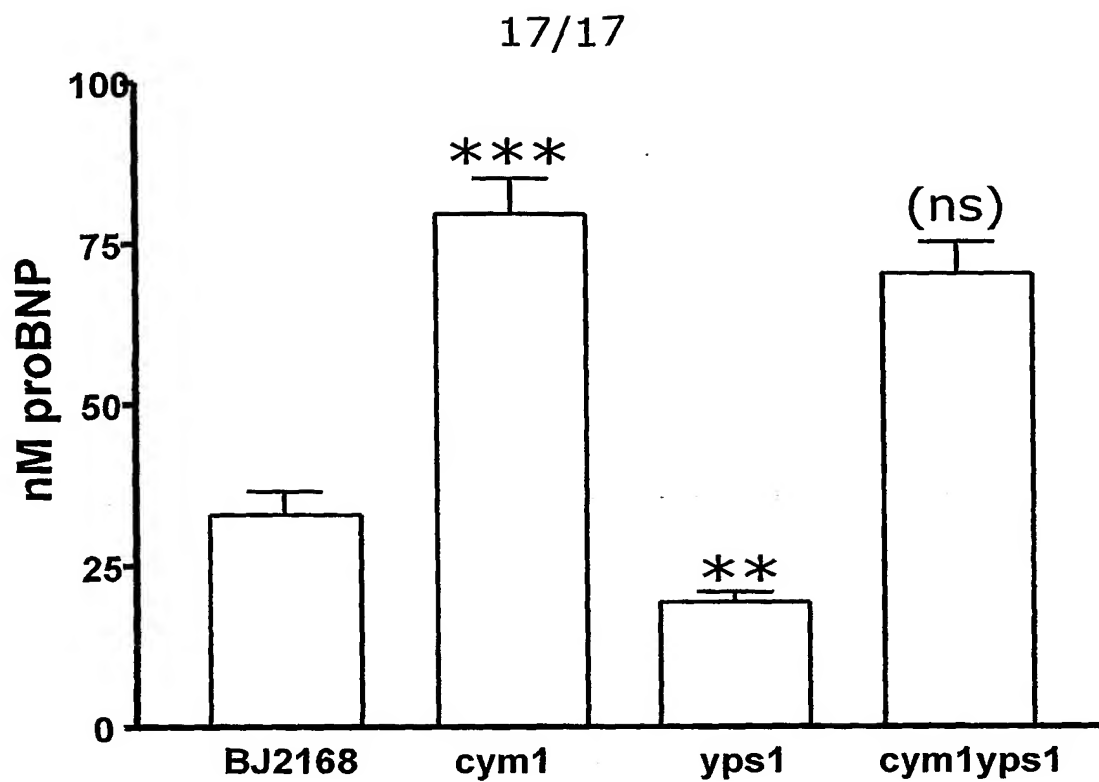


Fig. 15A

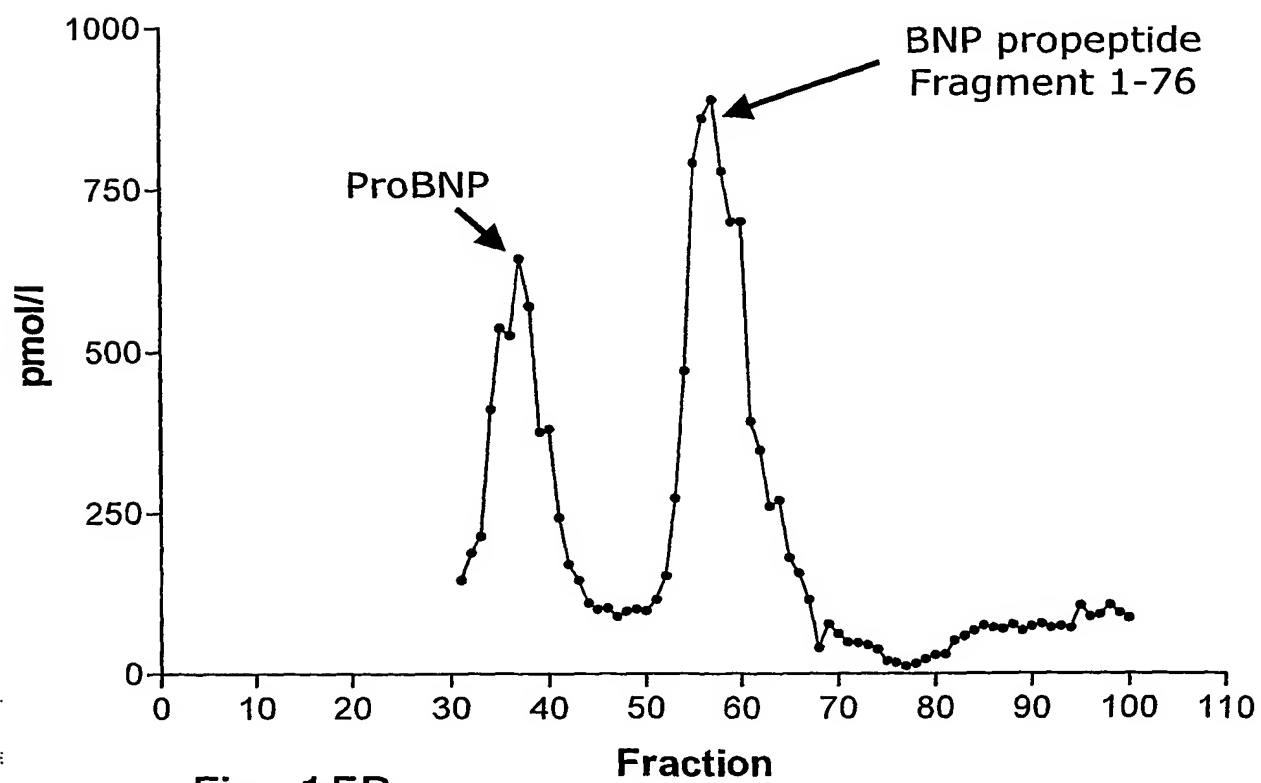


Fig. 15B

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Johnsen, Anders H.

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 40 Gln Gln His Leu Phe Gln Gly Thr Ala Tyr Gly Phe Asn Ser Gly Gly  
 210 215 220  
 45 Asp Pro Leu Ala Ile Pro Asp Leu Lys Tyr Glu Glu Leu Val Lys Phe  
 225 230 235 240  
 His Arg Ser His Tyr His Pro Ser Asn Ala Lys Ile Leu Ser Tyr Gly  
 245 250 255  
 50 Ser Phe Pro Leu Glu Asp Asn Leu Ser Ala Leu Ser Glu Thr Phe Arg  
 260 265 270  
 Pro Phe Ser Lys Arg Glu Leu Asn Leu Pro Asn Thr Phe Leu Lys Glu  
 275 280 285  
 55 Phe Asp Gln Glu Lys Arg Val Val Glu Tyr Gly Pro Leu Asp Pro Val  
 290 295 300



15/67

Met Ala Pro Gly Arg Gln Val Lys Thr Ser Ile Ser Phe Leu Ala Asn  
 305 310 315 320

5 Asp Thr Ser Asn Val Tyr Glu Thr Phe Ala Leu Lys Val Leu Ser Lys  
 325 330 335

10 Leu Cys Phe Asp Gly Phe Ser Ser Pro Phe Tyr Lys Ala Leu Ile Glu  
 340 345 350

15 Ser Gly Leu Gly Thr Asp Phe Ala Pro Asn Ser Gly Tyr Asp Ser Thr  
 355 360 365

20 Thr Lys Arg Gly Ile Phe Ser Val Gly Leu Glu Gly Ala Ser Glu Glu  
 370 375 380

Ser Leu Ala Lys Ile Glu Asn Leu Val Tyr Ser Ile Phe Asn Asp Leu  
 385 390 395 400

25 Ala Leu Lys Gly Phe Glu Asn Glu Lys Leu Glu Ala Ile Leu His Gln  
 405 410 415

30 Met Glu Ile Ser Leu Lys His Lys Ser Ala His Phe Gly Ile Gly Leu  
 420 425 430

35 Ala Gln Ser Leu Pro Phe Asn Trp Phe Asn Gly Ala Asp Pro Ala Asp  
 435 440 445

40 Trp Leu Ser Phe Asn Lys Gln Ile Glu Trp Leu Lys Gln Lys Asn Ser  
 450 455 460

Asp Gly Lys Leu Phe Gln Lys Leu Ile Lys Lys Tyr Ile Leu Glu Asn  
 465 470 475 480

45 Lys Ser Arg Phe Val Phe Thr Met Leu Pro Ser Ser Thr Phe Pro Gln  
 485 490 495

50 Arg Leu Gln Glu Ala Glu Ala Lys Lys Leu Gln Glu Arg Thr Ser Lys  
 500 505 510  
 Leu Thr Asp Glu Asp Ile Ala Glu Ile Glu Lys Thr Ser Val Lys Leu  
 515 520 525

55 Leu Glu Ala Gln Ser Thr Pro Ala Asp Thr Ser Cys Leu Pro Thr Leu  
 530 535 540

16/67

Ser Val Ser Asp Ile Pro Glu Thr Ile Asp Glu Thr Lys Leu Lys Phe  
 545 550 555 560

5 Leu Asp Ile Ala Gly Met Lys Ala Gln Trp Tyr Asp Leu Ala Ala Gly  
 565 570 575

10 Leu Thr Tyr Ile Arg Leu Leu Leu Pro Leu Lys Asn Phe Pro Glu Ser  
 580 585 590

15 Leu Ile Pro Tyr Leu Pro Val Tyr Cys Asp Ala Cys Leu Asn Leu Gly  
 595 600 605

20 Thr His Ser Glu Ser Ile Gly Asp Leu Glu His Gln Ile Arg Arg Tyr  
 610 615 620

Thr Gly Gly Ile Ser Ile Ser Pro Ser Ala Val Thr Asn Asn Ser Asp  
 625 630 635 640

25 Val Ser Lys Tyr Glu Leu Gly Ile Ala Ile Ser Gly Tyr Ala Leu Asp  
 645 650 655

30 Lys Asn Val Gly Lys Leu Val Glu Leu Ile Asn Lys Ala Phe Trp Asn  
 660 665 670

35 Thr Asn Leu Ser Asn Thr Asp Lys Leu Ala Ile Met Leu Lys Thr Ser  
 675 680 685

40 Val Ser Gly Ile Thr Asp Gly Ile Ala Glu Lys Gly His Ser Phe Ala  
 690 695 700

Lys Val Ser Ser Ala Ser Gly Leu Thr Glu Lys Thr Ser Ile Thr Glu  
 705 710 715 720

45 Gln Leu Gly Gly Leu Thr Gln Val Lys Leu Leu Ser Gln Leu Ser Arg  
 725 730 735

50 Glu Glu Ser Phe Gly Pro Leu Val Glu Lys Leu Thr Ala Ile Arg Glu  
 740 745 750  
 Ile Leu Arg Gly Thr Ser Gly Phe Lys Ala Ala Ile Asn Ala Ser Pro  
 755 760 765

55 Thr Gln His Glu Val Val Glu Lys Ala Leu Gln Lys Phe Met Lys Ser  
 770 775 780

Arg Gly Val Asn Gln Gln Thr Gln Thr Lys Ser Thr Ser Lys Glu Arg

17/67

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785                               790                               795                               800
5   Asn Gly Ile Asn Ser Ile Lys Thr Tyr His Glu Leu Pro Phe Gln Thr
    805                                810                                815

10  Tyr Phe Ala Ala Lys Ser Cys Leu Gly Val Pro Tyr Thr His Pro Asp
    820                                825                                830

15  Gly Ala Pro Leu Gln Ile Leu Ser Ser Leu Leu Thr His Lys Tyr Leu
    835                                840                                845

20  His Gly Glu Ile Arg Glu Lys Gly Gly Ala Tyr Gly Ala Gly Leu Ser
    850                                855                                860

25  Tyr Ser Gly Ile Asp Gly Val Leu Ser Phe Phe Thr Tyr Arg Asp Ser
    865                                870                                875                                880

    Asp Pro

30  <210> 6
    <211> 973
    <212> PRT
    <213> Clostridium perfringens

    <400> 6

35  Met Asn Phe Lys Glu Asn Asn Ile Tyr Ser Gly Phe Lys Leu Leu Asn
    1      5      10      15

40  Ile Glu Asn Leu Asn Glu Ile Gly Gly Val Gly Leu Arg Phe Glu His
    20      25      30

45  Glu Lys Thr Lys Ala Lys Leu Ile Lys Ile Leu Ser Glu Asp Asp Asn
    35      40      45

50  Lys Cys Phe Ala Ile Gly Phe Arg Thr Pro Pro Glu Asn Ser Thr Gly
    50      55      60

55  Val Pro His Ile Leu Glu His Ser Val Leu Cys Gly Ser Arg Lys Phe
    65      70      75      80

    Asn Thr Lys Glu Pro Phe Val Glu Leu Leu Lys Gly Ser Leu Asn Thr
    85      90      95

    Phe Leu Asn Ala Met Thr Tyr Pro Asp Lys Thr Ile Tyr Pro Val Ala
    100      105      110

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18/67

Ser Arg Asn Glu Lys Asp Phe Met Asn Leu Met Asp Val Tyr Leu Asp  
 115 120 125  
 5

Ala Val Leu Tyr Pro Asn Ile Tyr Lys His Lys Glu Ile Phe Met Gln  
 130 135 140  
 10

Glu Gly Trp His Tyr Tyr Ile Glu Asn Lys Glu Asp Glu Leu Lys Tyr  
 145 150 155 160  
 15

Asn Gly Val Val Tyr Asn Glu Met Lys Gly Ala Tyr Ser Ser Pro Asp  
 165 170 175  
 20

Ser Ile Leu Tyr Arg Lys Ile Pro Gln Thr Ile Tyr Pro Asp Thr Cys  
 180 185 190  
 25

Tyr Ala Leu Ser Ser Gly Gly Asp Pro Asp Glu Ile Pro Asn Leu Thr  
 195 200 205  
 30

Tyr Glu Glu Phe Val Glu Phe His Lys Lys Tyr Tyr His Pro Ser Asn  
 210 215 220  
 35

Phe Ile Asn Glu Glu Tyr Leu Lys Asn Phe Glu Tyr Lys Glu Ile Asp  
 245 250 255  
 40

Ser Glu Ile Lys Glu Gln Lys Ser Phe Glu Ser Met Lys Glu Glu Ser  
 260 265 270  
 45

Phe Thr Tyr Gly Ile Ala Glu Ser Glu Asp Leu Asn His Lys Ser Tyr  
 275 280 285  
 50

Tyr Ser Leu Asn Phe Val Ile Gly Asp Ala Thr Asp Gly Glu Lys Gly  
 290 295 300  
 Leu Ala Phe Asp Val Leu Ala Tyr Leu Leu Thr Arg Ser Thr Ala Ala  
 305 310 315 320  
 55

Pro Leu Lys Lys Ala Leu Ile Asp Ala Gly Ile Gly Lys Ala Val Ser  
 325 330 335  
 Gly Asp Phe Asp Asn Ser Thr Lys Gln Ser Ala Phe Thr Val Leu Val  
 340 345 350

19/67

Lys Asn Ala Glu Leu Asn Lys Glu Glu Glu Phe Lys Lys Val Val Met  
 355 360 365

5

Asp Thr Leu Lys Asp Leu Val Glu Asn Gly Ile Asp Lys Glu Leu Ile  
 370 375 380

10

Glu Ala Ser Ile Asn Arg Val Glu Phe Glu Leu Arg Glu Gly Asp Tyr  
 385 390 395 400

15

Gly Ser Tyr Pro Asn Gly Leu Ile Tyr Tyr Leu Lys Val Met Asp Ser  
 405 410 415

20

Trp Leu Tyr Asp Gly Asp Pro Tyr Val His Leu Glu Tyr Glu Lys Asn  
 420 425 430

25

Leu Glu Lys Ile Lys Ser Ala Leu Thr Ser Asn Tyr Phe Glu Asp Leu  
 435 440 445

30

Ile Glu Arg Tyr Met Ile Asn Asn Thr His Ser Ser Leu Val Ser Leu  
 450 455 460

35

His Pro Glu Lys Gly Ile Asn Glu Lys Lys Ser Ala Glu Leu Lys Lys  
 465 470 475 480

40

Lys Leu Glu Glu Ile Lys Asn Ser Phe Asp Glu Lys Thr Leu Asn Glu  
 485 490 495

45

Ile Ile Asp Asn Cys Lys Lys Leu Lys Glu Arg Gln Ser Thr Pro Asp  
 500 505 510

50

Lys Lys Glu Asp Leu Glu Ser Ile Pro Met Leu Ser Leu Glu Asp Ile  
 515 520 525

55

Asp Lys Glu Ala Thr Lys Ile Pro Thr Glu Glu Lys Glu Ile Asp Gly  
 530 535 540

Ile Thr Thr Leu His His Asp Phe His Thr Asn Lys Ile Asp Tyr Val  
 545 550 555 560

Asn Phe Phe Phe Asn Thr Asn Ser Val Pro Glu Asp Leu Ile Pro Tyr  
 565 570 575

Val Gly Leu Leu Cys Asp Ile Leu Gly Lys Cys Gly Thr Glu Asn Tyr  
 580 585 590

20/67

Asp Tyr Ser Lys Leu Ser Asn Ala Ile Asn Ile Ser Thr Gly Gly Ile  
 595 600 605

5 Ser Phe Gly Ala Ile Thr Phe Ala Asn Leu Lys Lys Asn Asn Glu Phe  
 610 615 620

10 Arg Pro Tyr Leu Glu Ile Ser Tyr Lys Ala Leu Ser Ser Lys Thr Asn  
 625 630 635 640

15 Lys Ala Ile Glu Leu Val Asp Glu Ile Val Asn His Thr Asp Leu Asp  
 645 650 655

Asp Met Asp Arg Ile Met Gln Ile Ile Arg Glu Lys Arg Ala Arg Leu  
 660 665 670

20 Glu Gly Ala Ile Phe Asp Ser Gly His Arg Ile Ala Met Lys Lys Val  
 675 680 685

25 Leu Ser Tyr Ser Thr Asn Arg Gly Ala Tyr Asp Glu Lys Ile Ser Gly  
 690 695 700

30 Leu Asp Tyr Tyr Asp Phe Leu Val Asn Ile Glu Lys Glu Asp Lys Lys  
 705 710 715 720

35 Ser Thr Ile Ser Asp Ser Leu Lys Lys Val Arg Asp Leu Ile Phe Asn  
 725 730 735

40 Lys Gly Asn Met Leu Ile Ser Tyr Ser Gly Lys Glu Glu Glu Tyr Glu  
 740 745 750

Asn Phe Lys Glu Lys Val Lys Tyr Leu Ile Ser Lys Thr Asn Asn Asn  
 755 760 765

45 Asp Phe Glu Lys Glu Glu Tyr Asn Phe Glu Leu Gly Lys Lys Asn Glu  
 770 775 780  
 Gly Leu Leu Thr Gln Gly Asn Val Gln Tyr Val Ala Lys Gly Gly Asn  
 785 790 795 800

50 Tyr Lys Thr His Gly Tyr Lys Tyr Ser Gly Ala Leu Ser Leu Leu Glu  
 805 810 815

55 Ser Ile Leu Gly Phe Asp Tyr Leu Trp Asn Ala Val Arg Val Lys Gly  
 820 825 830

Gly Ala Tyr Gly Val Phe Ser Asn Phe Arg Arg Asp Gly Gly Ala Tyr

21/67

835

840

845

5 Ile Val Ser Tyr Arg Asp Pro Asn Ile Lys Ser Thr Leu Glu Ala Tyr  
850 855 860

10 Asp Asn Ile Pro Lys Tyr Leu Asn Asp Phe Glu Ala Asp Glu Arg Glu  
865 870 875 880

15 Met Thr Lys Tyr Ile Ile Gly Thr Ile Arg Lys Tyr Asp Gln Pro Ile  
885 890 895

Ser Asn Gly Ile Lys Gly Asp Ile Ala Val Ser Tyr Tyr Leu Ser Asn  
900 905 910

20 Phe Thr Tyr Glu Asp Leu Gln Lys Glu Arg Glu Glu Ile Ile Asn Ala  
915 920 925

25 Asp Val Glu Lys Ile Lys Ser Phe Ala Pro Met Ile Lys Asp Leu Met  
930 935 940

30 Lys Glu Asp Tyr Ile Cys Val Leu Gly Asn Glu Glu Lys Ile Lys Glu  
945 950 955 960

Asn Lys Asp Leu Phe Asn Asn Ile Lys Ser Val Ile Lys  
965 970

35

&lt;210&gt; 7

&lt;211&gt; 971

&lt;212&gt; PRT

&lt;213&gt; Borrelia burgdorferi

40

&lt;400&gt; 7

45 Met Lys Lys Lys Lys Ile Phe Lys Leu Ile Ser Lys Thr Tyr Leu Glu  
1 5 10 15

Glu His Asp Ala Glu Gly Tyr Tyr Phe Lys His Glu Ser Gly Leu Glu  
20 25 30

50 Val Phe His Leu Lys Ser Asp Ser Phe Lys Glu Asn Ala Phe Cys Ile  
35 40 45

55 Ala Phe Lys Thr Ile Pro Ser Asn Asn Thr Gly Val Ala His Val Leu  
50 55 60

Glu His Thr Ile Phe Cys Gly Ser Ser Lys Tyr Lys Ile Lys Asp Pro  
65 70 75 80

22/67

Phe Leu Tyr Leu Leu Lys Gly Ser Leu Asn Thr Phe Leu Asn Ala Met  
 85 90 95  
 5

Thr Phe Pro Asp Lys Thr Ile Tyr Pro Ala Ala Ser Thr Ile Glu Lys  
 100 105 110  
 10

Asp Tyr Phe Asn Leu Phe Asn Ile Tyr Ala Asp Ser Ile Phe Asn Pro  
 115 120 125

15 Leu Leu Lys Lys Glu Ser Phe Met Gln Glu Gly Tyr Asn Ile Asn Pro  
 130 135 140

Lys Asp Phe Lys Val Ser Gly Ile Val Phe Asn Glu Met Lys Gly Ser  
 20 145 150 155 160

Tyr Ser Asn Lys Asn Ser Leu Ile Asn Glu Ile Val Ser Ser Ser Leu  
 165 170 175  
 25

Phe Glu Glu Gly Ala Tyr Lys Tyr Asp Ser Gly Gly Ile Pro Thr Asn  
 180 185 190  
 30

Ile Ile Asp Leu Thr Tyr Glu Ser Phe Leu Asp Phe Tyr Lys Lys Tyr  
 195 200 205

35 Tyr Thr Leu Glu Asn Cys Lys Ile Phe Leu Cys Gly Asn Thr Gln Thr  
 210 215 220

Glu Lys Asn Leu Asn Phe Ile Glu Lys Tyr Ile Ile Arg Pro Tyr Lys  
 40 225 230 235 240

Lys Glu Lys Ser Asn Val Asn Ile Asn Ile Glu Asn Val Lys Arg Trp  
 245 250 255  
 45 Glu Lys Gly Lys Lys Leu Thr Tyr Lys Ile Pro Lys Glu Asn Asp Asn  
 260 265 270

Ser Leu Gly Val Tyr Thr Ile Asn Trp Leu Cys Thr Glu Ile Asn Asn  
 50 275 280 285

Ile Glu Asp Ser Ile Gly Leu Glu Ile Leu Ser Glu Ile Leu Leu Asp  
 290 295 300  
 55

Asp Ser Cys Ser Phe Thr Ile Asn Ile Leu Lys Ser Gly Ile Gly Glu  
 305 310 315 320



23/67

Asp Ile Ala His Ile Ser Gly Ile Asn Thr Asp Leu Lys Glu Ser Ile  
 325 330 335

5  
 Phe Ser Phe Gly Leu Gln Asn Val Val Glu Asn Lys Glu Lys Glu Phe  
 340 345 350

10 Lys Asn Leu Val Phe Ser Glu Leu Lys Asn Leu Val Lys Asn Lys Ile  
 355 360 365

15 Pro Lys Glu Leu Ile Lys Gly Ile Leu Phe Gly Tyr Glu Phe Ala Leu  
 370 375 380

20 Lys Glu Glu Lys Gly Gln Asn Phe Pro Ile Ala Leu Met Ile Lys Ser  
 385 390 395 400

Phe Lys Gly Trp Leu Asn Gly Leu His Pro Ile Lys Thr Leu Gln Thr  
 405 410 415

25 Ser Tyr Tyr Ile Asn Glu Ile Thr Asn Lys Leu Glu Lys Gly Ile Tyr  
 420 425 430

30 Tyr Phe Glu Asn Leu Ile Glu Lys Tyr Leu Ile Phe Asn Asn His Tyr  
 435 440 445

35 Thr Leu Ile Ser Phe Ile Pro Ser His Asp Thr Glu Lys Glu Met Glu  
 450 455 460

40 Glu Glu Ile Glu Lys Lys Leu Met Ala Arg Glu Ile Glu Ile Lys Gln  
 465 470 475 480

Asn Pro Glu Glu Phe Leu Gln Phe Lys Lys Asp Tyr Asn Gln Phe Lys  
 485 490 495

45 Lys Tyr Gln Asn Lys Lys Asp Ser Lys Ala Asp Ile Ala Lys Leu Pro  
 500 505 510

50 Leu Leu Lys Ile Glu Asp Leu Pro Lys Gln Ile Glu Lys Ser Leu Asp  
 515 520 525

Leu Asn Glu Ile Lys Glu Leu Asn Leu His Ser Phe Lys Phe Lys Ser  
 530 535 540

55 Asn Asn Ile Phe Asn Val Asn Leu Phe Phe Lys Leu Asp Phe Leu Glu  
 545 550 555 560

24/67

Lys Glu Asp Tyr Ile Tyr Leu Ser Leu Phe Lys Arg Ala Leu Gln Asp  
 565 570 575

5 Leu Ser Thr Lys Asn Tyr Ser Tyr Ile Asn Ile Asn Asn Lys Ile Gln  
 580 585 590

10 Asn Thr Leu Gly Gln Ile Asn Ile Ser Glu Ser Tyr Asp Glu Asp Ile  
 595 600 605

15 Asp Gly Asn Ile Leu Asn Ser Phe Asn Ile Ser Phe Lys Ser Phe Asn  
 610 615 620

Asn Lys Val Lys Glu Ser Phe Glu Leu Ile Lys Glu Ile Leu Ile Asn  
 625 630 635 640

20 Ile Asn Phe His Asp Tyr Glu Arg Leu Lys Glu Ile Thr Leu Ser Leu  
 645 650 655

25 Lys Asn Asp Phe Lys Ser Leu Leu Ile Pro Lys Gly His Leu Leu Ala  
 660 665 670

30 Met Leu Arg Ser Lys Ser Lys Leu Lys Leu Asn Glu Tyr Leu Lys Glu  
 675 680 685

35 Leu Gln Asn Gly Ile Thr Gly Arg Glu Phe Trp Gln Lys Ala Lys Thr  
 690 695 700

Asp Thr Glu Ser Leu Lys Glu Ile Ala Asn Lys Leu Asp Asn Leu Lys  
 705 710 715 720

40 Asn Lys Ile Ile Leu Lys Asn Asn Leu Ser Ala Leu Ile Met Gly Asn  
 725 730 735

Thr Asp Asp Ile Leu Lys Asn Leu Glu Asn Glu Phe Phe Asn Leu Lys  
 740 745 750

45 Glu Ser Leu Glu Glu Ser Asn His Tyr Asn Gly Leu Leu Asn Leu Asp  
 755 760 765

50 Ala Asn Ser Lys Ala Leu Arg Glu Ile Ile Ile Ile Gln Ser Lys Val  
 770 775 780

55 Ala Phe Asn Ala Ile Cys Phe Pro Ser Tyr Lys Ile Asn Asp Glu Asn  
 785 790 795 800

Tyr Pro Lys Ala Asn Phe Leu Glu His Val Leu Arg Ser Gly Ile Phe

25/67

805

810

815

5 Trp Glu Lys Ile Arg Val Met Gly Gly Ala Tyr Gly Ala Ser Ala Ser  
820 825 830

10 Ile Ala Asn Gly Ile Phe Ser Phe Ala Ser Tyr Arg Asp Pro Asn Phe  
835 840 845

15 Thr Lys Thr Tyr Gln Ala Phe Glu Lys Ser Leu Glu Glu Leu Ala Asn  
850 855 860

Asn Lys Met Thr Asp Asp Glu Ile Tyr Thr Tyr Leu Ile Gly Leu Ile  
865 870 875 880

20 Gly Thr Asn Ile Tyr Val Lys Thr Lys Ala Thr Glu Ala Leu Gln Ser  
885 890 895

25 Tyr Arg Arg Lys Met Leu Asn Ile Ser Asp Ser Leu Arg Gln Asp Ile  
900 905 910

30 Arg Asn Ala Tyr Phe Thr Ile Thr Pro Gln Asp Ile Lys Glu Ile Ser  
915 920 925

Thr Lys Ile Leu Thr Gln Ile Arg Gln His Asn Ser Ile Ala Ser Leu  
930 935 940

35 Val Asn Asn Gln Ile Tyr Glu Glu Glu Lys Asn Asn Leu Glu Lys Leu  
945 950 955 960

40 Ile Gly Lys Glu Tyr Ser Leu Lys Lys Ile Tyr  
965 970

<210> 8

<211> 995

<212> PRT

45 <213> Caenorhabditis elegans

<400> 8

50 Met Ser Ala Ser Lys Leu Trp Ser Cys Thr Glu Thr Val Leu Asn Gly  
1 5 10 15

55 Gly Ile Lys Leu Phe Leu Tyr Ser Ser Lys Asn Thr Lys Leu Arg Val  
20 25 30

Ala Ile Gly Glu Val Pro Gly Pro Met Val His Gly Ala Val Ser Phe  
35 40 45

26/67

Val Thr Glu Ala Asp Ser Asp Asp Gly Leu Pro His Thr Leu Glu His  
 50 55 60

5  
 Leu Val Phe Met Gly Ser Lys Lys Tyr Pro Phe Lys Gly Val Leu Asp  
 65 70 75 80

10 Val Ile Ala Asn Arg Cys Leu Ala Asp Gly Thr Asn Ala Trp Thr Asp  
 85 90 95

15 Thr Asp His Thr Ala Tyr Thr Leu Ser Thr Val Gly Ser Asp Gly Phe  
 100 105 110

20 Leu Lys Val Leu Pro Val Tyr Ile Asn His Leu Leu Thr Pro Met Leu  
 115 120 125

Thr Ala Ser Gln Phe Ala Thr Glu Val His His Ile Thr Gly Glu Gly  
 130 135 140

25  
 Asn Asp Ala Gly Val Val Tyr Ser Glu Met Gln Asp His Glu Ser Glu  
 145 150 155 160

30 Met Glu Ser Ile Met Asp Arg Lys Thr Lys Glu Val Ile Tyr Pro Pro  
 165 170 175

35 Phe Asn Pro Tyr Ala Val Asp Thr Gly Gly Arg Leu Lys Asn Leu Arg  
 180 185 190

40 Glu Ser Cys Thr Leu Glu Lys Val Arg Asp Tyr His Lys Lys Phe Tyr  
 195 200 205

His Leu Ser Asn Met Val Val Thr Val Cys Gly Met Val Asp His Asp  
 210 215 220

45 Gln Val Leu Glu Ile Met Asn Asn Val Glu Asn Glu His Met Ser Thr  
 225 230 235 240

50 Val Pro Asp His Phe Pro Lys Pro Phe Ser Phe Ala Leu Ser Asp Ile  
 245 250 255

55 Lys Glu Ser Thr Val His Arg Val Glu Cys Pro Thr Asp Asp Ala Ser  
 260 265 270

Arg Gly Ala Val Glu Val Ala Trp Phe Ala His Ser Pro Ser Asp Leu  
 275 280 285

27/67

Glu Thr His Ser Ser Leu His Val Leu Phe Asp Tyr Leu Ser Asn Thr  
 290 295 300  
 5  
 Ser Val Ala Pro Leu Gln Lys Asp Phe Ile Leu Leu Glu Asp Pro Leu  
 305 310 315 320  
 10 Ala Ser Ser Val Ser Phe His Ile Ala Glu Gly Val Arg Cys Asp Leu  
 325 330 335  
 15 Arg Leu Asn Phe Ala Gly Val Pro Val Glu Lys Leu Asp Glu Cys Ala  
 340 345 350  
 20 Pro Lys Phe Phe Asp Lys Thr Val Arg Glu His Leu Glu Glu Ala Asn  
 355 360 365  
 Phe Asp Met Glu Arg Met Gly Tyr Leu Ile Asp Gln Thr Ile Leu Asn  
 370 375 380  
 25 Glu Leu Val Lys Leu Glu Thr Asn Ala Pro Lys Asp Ile Met Ser His  
 385 390 395 400  
 30 Ile Ile Gly His Gln Leu Phe Asp Asn Glu Asp Glu Glu Leu Phe Lys  
 405 410 415  
 35 Lys Arg Thr Asn Glu Ile Asp Phe Leu Lys Lys Leu Lys Ser Glu Pro  
 420 425 430  
 40 Ala Ser Tyr Trp Val Gln Leu Val Asn Lys Tyr Phe Thr Ala Pro Ser  
 435 440 445  
 Ala Thr Val Ile Gly Val Pro Asn Glu Glu Leu Val Asp Lys Ile Ala  
 450 455 460  
 45 Glu Glu Glu Glu Lys Arg Ile Ala Ala Gln Cys Glu Lys Leu Gly Lys  
 465 470 475 480  
 50 Lys Gly Leu Glu Glu Ala Gly Lys Ser Leu Glu Ala Ala Ile Leu Glu  
 485 490 495  
 Asn Thr Ala Asn His Pro Ser Ala Glu Leu Leu Asp Gln Leu Ile Val  
 500 505 510  
 55 Lys Asp Leu Glu Ala Phe Asp Arg Phe Pro Val Gln Ser Leu Thr Ser  
 515 520 525

28/67

Asn Ser Pro Ser Leu Thr Pro Gln Gln Ser Thr Phe Leu Ala Gln Phe  
 530 535 540

5 Pro Phe His Ala Asn Leu His Asn Cys Pro Thr Lys Phe Val Glu Ile  
 545 550 555 560

10 Phe Phe Leu Leu Asp Ser Ser Asn Leu Ser Ile Glu Asp Arg Ser Tyr  
 565 570 575

15 Leu Phe Leu Tyr Thr Asp Leu Leu Phe Glu Ser Pro Ala Met Ile Asp  
 580 585 590

Gly Val Leu Thr Ser Ala Asp Asp Val Ala Lys His Phe Thr Lys Asp  
 595 600 605

20 Leu Ile Asp His Ser Ile Gln Val Gly Val Ser Gly Leu Tyr Asp Arg  
 610 615 620

25 Phe Val Asn Leu Arg Ile Lys Val Gly Ala Asp Lys Tyr Pro Leu Leu  
 625 630 635 640

30 Ala Lys Trp Ala Gln Ile Phe Thr Gln Gly Val Val Phe Asp Pro Ser  
 645 650 655

35 Arg Ile His Gln Cys Ala Gln Lys Leu Ala Gly Glu Ala Arg Asp Arg  
 660 665 670

Lys Arg Asp Gly Cys Thr Val Ala Ser Thr Ala Val Ala Ser Met Val  
 675 680 685

40 Tyr Gly Lys Asn Thr Asn Cys Ile Leu Phe Asp Glu Leu Val Leu Glu  
 690 695 700

45 Lys Leu His Glu Lys Ile Ser Lys Asp Val Met Lys Asn Pro Glu Ala  
 705 710 715 720

Val Leu Glu Lys Leu Glu Gln Val Arg Ser Ala Leu Phe Ser Asn Gly  
 725 730 735

50 Val Asn Ala His Phe Val Ala Asp Val Asp Ser Ile Asp Pro Lys Met  
 740 745 750

55 Leu Ser Ser Asp Leu Trp Thr Trp Val Gln Ala Asp Pro Arg Phe Gly  
 755 760 765

Pro Gly His Gln Phe Ser Ala Glu Ala Gly Glu Asn Val Ser Leu Glu

29/67

770

775

780

Leu Gly Lys Glu Leu Leu Ile Gly Val Gly Gly Ser Glu Ser Ser Phe  
 5 785 790 795 800

Ile Tyr Gln Thr Ser Phe Leu Asp Ala Asn Trp Asn Ser Glu Glu Leu  
 10 805 810 815

Ile Pro Ala Met Ile Phe Gly Gln Tyr Leu Ser Gln Cys Glu Gly Pro  
 820 825 830

15 Leu Trp Arg Ala Ile Arg Gly Asp Gly Leu Ala Tyr Gly Ala Asn Val  
 835 840 845

20 Phe Val Lys Pro Asp Arg Lys Gln Ile Thr Leu Ser Leu Tyr Arg Cys  
 850 855 860

25 Ala Gln Pro Ala Val Ala Tyr Glu Arg Thr Arg Asp Ile Ile Arg Lys  
 865 870 875 880

Ile Val Glu Ser Gly Glu Ile Ser Lys Ala Glu Phe Glu Gly Ala Lys  
 30 885 890 895

Arg Ser Thr Val Phe Glu Met Met Lys Arg Glu Gly Thr Val Ser Gly  
 900 905 910

35 Ala Ala Lys Ile Ser Ile Leu Asn Asn Phe Arg Gln Thr Pro His Pro  
 915 920 925

Phe Asn Ile Asp Leu Cys Arg Arg Ile Trp Asn Leu Thr Ser Glu Glu  
 40 930 935 940

Met Val Lys Ile Gly Gly Pro Pro Leu Ala Arg Leu Phe Asp Glu Lys  
 945 950 955 960

45 Cys Phe Val Arg Ser Ile Ala Val His Pro Ser Lys Leu Asn Glu Met  
 965 970 975

50 Lys Lys Ala Phe Pro Gly Ser Ser Lys Ile Lys Ile Ser Asp Leu Gln  
 980 985 990

55 Phe Ala Cys  
 995

&lt;210&gt; 9

&lt;211&gt; 962

30/67

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 9

5

Met Pro Arg Ser Thr Trp Phe Lys Ala Leu Leu Leu Leu Val Ala Leu  
 1 5 10 15

10 Trp Ala Pro Leu Ser Gln Ala Glu Thr Gly Trp Gln Pro Ile Gln Glu  
 20 25 30

15 Thr Ile Arg Lys Ser Asp Lys Asp Asn Arg Gln Tyr Gln Ala Ile Arg  
 35 40 45

20 Leu Asp Asn Gly Met Val Val Leu Leu Val Ser Asp Pro Gln Ala Val  
 50 55 60

Lys Ser Leu Ser Ala Leu Val Val Pro Val Gly Ser Leu Glu Asp Pro  
 65 70 75 80

25

Glu Ala Tyr Gln Gly Leu Ala His Tyr Leu Glu His Met Ser Leu Met  
 85 90 95

30 Gly Ser Lys Lys Tyr Pro Gln Ala Asp Ser Leu Ala Glu Tyr Leu Lys  
 100 105 110

35 Met His Gly Gly Ser His Asn Ala Ser Thr Ala Pro Tyr Arg Thr Ala  
 115 120 125

Phe Tyr Leu Glu Val Glu Asn Asp Ala Leu Pro Gly Ala Val Asp Arg  
 130 135 140

40

Leu Ala Asp Ala Ile Ala Glu Pro Leu Leu Asp Lys Lys Tyr Ala Glu  
 145 150 155 160

45 Arg Glu Arg Asn Ala Val Asn Ala Glu Leu Thr Met Ala Arg Thr Arg  
 165 170 175

50 Asp Gly Met Arg Met Ala Gln Val Ser Ala Glu Thr Ile Asn Pro Ala  
 180 185 190

His Pro Gly Ser Lys Phe Ser Gly Gly Asn Leu Glu Thr Leu Ser Asp  
 195 200 205

55

Lys Pro Gly Asn Pro Val Gln Gln Ala Leu Lys Asp Phe His Glu Lys  
 210 215 220



31/67

Tyr Tyr Ser Ala Asn Leu Met Lys Ala Val Ile Tyr Ser Asn Lys Pro  
225 230 235 240

5  
Leu Pro Glu Leu Ala Lys Met Ala Ala Asp Thr Phe Gly Arg Val Pro  
245 250 255

10 Asn Lys Glu Ser Lys Lys Pro Glu Ile Thr Val Pro Val Val Thr Asp  
260 265 270

15 Ala Gln Lys Gly Ile Ile Ile His Tyr Val Pro Ala Leu Pro Arg Lys  
275 280 285

20 Val Leu Arg Val Glu Phe Arg Ile Asp Asn Asn Ser Ala Lys Phe Arg  
290 295 300

Ser Lys Thr Asp Glu Leu Ile Thr Tyr Leu Ile Gly Asn Arg Ser Pro  
305 310 315 320

25 Gly Thr Leu Ser Asp Trp Leu Gln Lys Gln Gly Leu Val Glu Gly Ile  
325 330 335

30 Ser Ala Asn Ser Asp Pro Ile Val Asn Gly Asn Ser Gly Val Leu Ala  
340 345 350

35 Ile Ser Ala Ser Leu Thr Asp Lys Gly Leu Ala Asn Arg Asp Gln Val  
355 360 365  
Val Ala Ala Ile Phe Ser Tyr Leu Asn Leu Leu Arg Glu Lys Gly Ile  
370 375 380

40 Asp Lys Gln Tyr Phe Asp Glu Leu Ala Asn Val Leu Asp Ile Asp Phe  
385 390 395 400

45 Arg Tyr Pro Ser Ile Thr Arg Asp Met Asp Tyr Val Glu Trp Leu Ala  
405 410 415

50 Asp Thr Met Ile Arg Val Pro Val Glu His Thr Leu Asp Ala Val Asn  
420 425 430

Ile Ala Asp Arg Tyr Asp Ala Lys Ala Val Lys Glu Arg Leu Ala Met  
435 440 445

55 Met Thr Pro Gln Asn Ala Arg Ile Trp Tyr Ile Ser Pro Lys Glu Pro  
450 455 460

32/67

His Asn Lys Thr Ala Tyr Phe Val Asp Ala Pro Tyr Gln Val Asp Lys  
 465 470 475 480

5 Ile Ser Ala Gln Thr Phe Ala Asp Trp Gln Lys Lys Ala Ala Asp Ile  
 485 490 495

10 Ala Leu Ser Leu Pro Glu Leu Asn Pro Tyr Ile Pro Asp Asp Phe Ser  
 500 505 510

15 Leu Ile Lys Ser Glu Lys Lys Tyr Asp His Pro Glu Leu Ile Val Asp  
 515 520 525

20 Glu Ser Asn Leu Arg Val Val Tyr Ala Pro Ser Arg Tyr Phe Ala Ser  
 530 535 540

25 Glu Pro Lys Ala Asp Val Ser Leu Ile Leu Arg Asn Pro Lys Ala Met  
 545 550 555 560

30 Asp Ser Ala Arg Asn Gln Val Met Phe Ala Leu Asn Asp Tyr Leu Ala  
 565 570 575

35 Gly Leu Ala Leu Asp Gln Leu Ser Asn Gln Ala Ser Val Gly Gly Ile  
 580 585 590

40 Ser Phe Ser Thr Asn Ala Asn Asn Gly Leu Met Val Asn Ala Asn Gly  
 595 600 605

45 Tyr Thr Gln Arg Leu Pro Gln Leu Phe Gln Ala Leu Leu Glu Gly Tyr  
 610 615 620

50 Phe Ser Tyr Thr Ala Thr Glu Asp Gln Leu Glu Gln Ala Lys Ser Trp  
 625 630 635 640

55 Tyr Asn Gln Met Met Asp Ser Ala Glu Lys Gly Lys Ala Phe Glu Gln  
 645 650 655

60 Ala Ile Met Pro Ala Gln Met Leu Ser Gln Val Pro Tyr Phe Ser Arg  
 660 665 670

65 Asp Glu Arg Arg Lys Ile Leu Pro Ser Ile Thr Leu Lys Glu Val Leu  
 675 680 685

70 Ala Tyr Arg Asp Ala Leu Lys Ser Gly Ala Arg Pro Glu Phe Met Val  
 690 695 700

Ile Gly Asn Met Thr Glu Ala Gln Ala Thr Thr Leu Ala Arg Asp Val

33/67

	705		710		715		720
5	Gln Lys Gln Leu Gly Ala Asp Gly Ser Glu Trp Cys Arg Asn Lys Asp	725		730		735	
10	Val Val Val Asp Lys Lys Gln Ser Val Ile Phe Glu Lys Ala Gly Asn	740		745		750	
	Ser Thr Asp Ser Ala Leu Ala Ala Val Phe Val Pro Thr Gly Tyr Asp	755		760		765	
15	Glu Tyr Thr Ser Ser Ala Tyr Ser Ser Leu Leu Gly Gln Ile Val Gln	770		775		780	
20	Pro Trp Phe Tyr Asn Gln Leu Arg Thr Glu Glu Gln Leu Gly Tyr Ala	785		790		795	800
25	Val Phe Ala Phe Pro Met Ser Val Gly Arg Gln Trp Gly Met Gly Phe	805		810		815	
30	Leu Leu Gln Ser Asn Asp Lys Gln Pro Ser Phe Leu Trp Glu Arg Tyr	820		825		830	
	Lys Ala Phe Phe Pro Thr Ala Glu Ala Lys Leu Arg Ala Met Lys Pro	835		840		845	
35	Asp Glu Phe Ala Gln Ile Gln Gln Ala Val Ile Thr Gln Met Leu Gln	850		855		860	
40	Ala Pro Gln Thr Leu Gly Glu Glu Ala Ser Lys Leu Ser Lys Asp Phe	865		870		875	880
	Asp Arg Gly Asn Met Arg Phe Asp Ser Arg Asp Lys Ile Val Ala Gln	885		890		895	
45	Ile Lys Leu Leu Thr Pro Gln Lys Leu Ala Asp Phe Phe His Gln Ala	900		905		910	
50	Val Val Glu Pro Gln Gly Met Ala Ile Leu Ser Gln Ile Ser Gly Ser	915		920		925	
55	Gln Asn Gly Lys Ala Glu Tyr Val His Pro Glu Gly Trp Lys Val Trp	930		935		940	
	Glu Asn Val Ser Ala Leu Gln Gln Thr Met Pro Leu Met Ser Glu Lys	945		950		955	960

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Asn Glu

5

&lt;210&gt; 10

&lt;211&gt; 1161

&lt;212&gt; PRT

10 &lt;213&gt; Homo sapiens

&lt;400&gt; 10

Met Leu Arg Arg Val Ala Val Ala Ala Val Phe Ala Thr Gly Arg Lys  
 15 1 5 10 15

Leu Arg Cys Glu Ala Gly Arg Asp Val Thr Ala Val Gly Arg Ile Glu  
 20 20 25 30

Ala Arg Gly Leu Cys Glu Glu Ser Ala Lys Pro Phe Pro Thr Leu Thr  
 35 40 45

25

Met Pro Gly Arg Asn Lys Ala Lys Ser Thr Cys Ser Cys Pro Asp Leu  
 50 55 60

Gln Pro Asn Gly Gln Asp Leu Gly Glu Ser Gly Arg Val Ala Arg Leu  
 65 70 75 80  
 Gly Ala Asp Glu Ser Glu Glu Glu Gly Arg Ser Leu Ser Asn Val Gly  
 85 90 95

35

Asp Pro Glu Ile Ile Lys Ser Pro Ser Asp Pro Lys Gln Tyr Arg Tyr  
 100 105 110

Ile Lys Leu Gln Asn Gly Leu Gln Ala Leu Leu Ile Ser Asp Leu Ser  
 115 120 125

Asn Val Glu Gly Lys Thr Gly Asn Ala Thr Asp Glu Glu Glu Glu  
 45 130 135 140

Glu Glu Glu Glu Glu Glu Gly Glu Glu Glu Glu Glu Glu Glu Asp  
 145 150 155 160

50

Asp Asp Asp Asp Asp Asp Glu Asp Ser Gly Ala Glu Ile Gln Asp Asp  
 165 170 175

55

Asp Glu Glu Gly Phe Asp Asp Glu Glu Glu Phe Asp Asp Asp Glu His  
 180 185 190

35/67

Asp Asp Asp Asp Leu Asp Asn Glu Glu Asn Glu Leu Glu Glu Leu Glu  
 195 200 205

5 Glu Arg Val Glu Ala Arg Lys Lys Thr Thr Glu Lys Gln Ser Ala Ala  
 210 215 220

10 Ala Leu Cys Val Gly Val Gly Ser Phe Ala Asp Pro Asp Asp Leu Pro  
 225 230 235 240

15 Gly Leu Ala His Phe Leu Glu His Met Val Phe Met Gly Ser Leu Lys  
 245 250 255

Tyr Pro Asp Glu Asn Gly Phe Asp Ala Phe Leu Lys Lys His Gly Gly  
 260 265 270

20 Ser Asp Asn Ala Ser Thr Asp Cys Glu Arg Thr Val Phe Gln Phe Asp  
 275 280 285

25 Val Gln Arg Lys Tyr Phe Lys Glu Ala Leu Asp Arg Trp Ala Gln Phe  
 290 295 300

30 Phe Ile His Pro Leu Met Ile Arg Asp Ala Ile Asp Arg Glu Val Glu  
 305 310 315 320  
 Ala Val Asp Ser Glu Tyr Gln Leu Ala Arg Pro Ser Asp Ala Asn Arg  
 325 330 335

35 Lys Glu Met Leu Phe Gly Ser Leu Ala Arg Pro Gly His Pro Met Gly  
 340 345 350

40 Lys Phe Phe Trp Gly Asn Ala Glu Thr Leu Lys His Glu Pro Lys Lys  
 355 360 365

45 Asn Asn Ile Asp Thr His Ala Arg Leu Arg Glu Phe Trp Met Arg Tyr  
 370 375 380

Tyr Ser Ala His Tyr Met Thr Leu Val Val Gln Ser Lys Glu Thr Leu  
 385 390 395 400

50 Asp Thr Leu Glu Lys Trp Val Thr Glu Ile Phe Ser Gln Ile Pro Asn  
 405 410 415

55 Asn Gly Leu Pro Lys Pro Asn Phe Ser His Leu Thr Asp Pro Phe Asp  
 420 425 430

Thr Pro Ala Phe Asn Lys Leu Tyr Arg Val Val Pro Ile Arg Lys Ile

36/67

435

440

445

5 His Ala Leu Thr Ile Thr Trp Ala Leu Pro Pro Gln Gln Gln His Tyr  
 450 455 460

10 Arg Val Lys Pro Leu His Tyr Ile Ser Trp Leu Val Gly His Glu Gly  
 465 470 475 480

15 Lys Gly Ser Ile Leu Ser Tyr Leu Arg Lys Lys Cys Trp Ala Leu Ala  
 485 490 495

20 Leu Phe Gly Gly Asn Gly Glu Thr Gly Phe Glu Gln Asn Ser Thr Tyr  
 500 505 510

25 Ser Val Phe Ser Ile Ser Ile Thr Leu Thr Asp Glu Gly Tyr Glu His  
 515 520 525

30 Phe Tyr Glu Val Ala His Thr Val Phe Gln Tyr Leu Lys Met Leu Gln  
 530 535 540

35 Lys Leu Gly Pro Glu Lys Arg Val Phe Glu Glu Ile Gln Lys Ile Glu  
 545 550 555 560

40 Asp Asn Glu Phe His Tyr Gln Glu Gln Thr Asp Pro Val Glu Tyr Val  
 565 570 575

45 Glu Asn Met Cys Glu Asn Met Gln Leu Tyr Pro Arg Gln Asp Phe Leu  
 580 585 590

50 Thr Gly Asp Gln Leu Leu Phe Glu Tyr Lys Pro Glu Val Ile Ala Glu  
 595 600 605

55 Ala Leu Asn Gln Leu Val Pro Gln Lys Ala Asn Leu Val Leu Leu Ser  
 610 615 620

60 Gly Ala Asn Glu Gly Arg Cys Asp Leu Lys Glu Lys Trp Phe Gly Thr  
 625 630 635 640

65 Gln Tyr Ser Ile Glu Asp Ile Glu Asn Ser Trp Thr Glu Leu Trp Lys  
 645 650 655

70 Ser Asn Phe Asp Leu Asn Ser Asp Leu His Leu Pro Ala Glu Asn Lys  
 660 665 670

75 Tyr Ile Ala Thr Asp Phe Thr Leu Lys Ala Phe Asp Cys Pro Glu Thr  
 675 680 685

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	Glu	Tyr	Pro	Ala	Lys	Ile	Val	Asn	Thr	Pro	Gln	Gly	Cys	Leu	Trp	Tyr	
	690						695					700					
5																	
	Lys	Lys	Asp	Asn	Lys	Phe	Lys	Ile	Pro	Lys	Ala	Tyr	Ile	Arg	Phe	His	
	705					710					715					720	
10																	
	Leu	Ile	Ser	Pro	Leu	Ile	Gln	Lys	Ser	Ala	Ala	Asn	Val	Val	Leu	Phe	
					725					730					735		
15																	
	Asp	Ile	Phe	Val	Asn	Ile	Leu	Thr	His	Asn	Leu	Ala	Glu	Pro	Ala	Tyr	
				740					745					750			
20																	
	Glu	Ala	Asp	Val	Ala	Gln	Leu	Glu	Tyr	Lys	Leu	Val	Ala	Gly	Glu	His	
			755					760					765				
25																	
	Gly	Leu	Ile	Ile	Arg	Val	Lys	Gly	Phe	Asn	His	Lys	Leu	Pro	Leu	Leu	
	770						775					780					
30																	
	Phe	Gln	Leu	Ile	Ile	Asp	Tyr	Leu	Thr	Glu	Phe	Ser	Ser	Thr	Pro	Ala	
	785					790					795					800	
	Val	Phe	Thr	Met	Ile	Thr	Glu	Gln	Leu	Lys	Lys	Thr	Tyr	Phe	Asn	Ile	
					805					810					815		
35																	
	Leu	Ile	Lys	Pro	Glu	Thr	Leu	Ala	Lys	Asp	Val	Arg	Leu	Leu	Ile	Leu	
				820					825					830			
40																	
	Glu	Tyr	Ser	Arg	Trp	Ser	Met	Ile	Asp	Lys	Tyr	Arg	Ala	Leu	Met	Asp	
			835					840					845				
45																	
	Gly	Leu	Ser	Leu	Glu	Ser	Leu	Leu	Asn	Phe	Val	Lys	Asp	Phe	Lys	Ser	
	850						855					860					
50																	
	Gln	Leu	Phe	Val	Glu	Gly	Leu	Val	Gln	Gly	Asn	Val	Thr	Ser	Thr	Glu	
	865					870					875					880	
55																	
	Ser	Met	Asp	Phe	Leu	Arg	Tyr	Val	Val	Asp	Lys	Leu	Asn	Phe	Val	Pro	
					885					890					895		
60																	
	Leu	Glu	Arg	Glu	Met	Pro	Val	Gln	Phe	Gln	Val	Val	Glu	Leu	Pro	Ser	
				900					905					910			
65																	
	Gly	His	His	Leu	Cys	Lys	Val	Arg	Ala	Leu	Asn	Lys	Gly	Asp	Ala	Asn	
		915						920									

38/67

Ser Glu Val Thr Val Tyr Tyr Gln Ser Gly Thr Arg Ser Leu Arg Glu  
 930 935 940

5

Tyr Thr Leu Met Glu Leu Leu Val Met His Met Glu Glu Pro Cys Phe  
 945 950 955 960

10 Asp Phe Leu Arg Thr Lys Gln Thr Leu Gly Tyr His Val Tyr Pro Thr  
 965 970 975

15 Cys Arg Asn Thr Ser Gly Ile Leu Gly Phe Ser Val Thr Val Gly Thr  
 980 985 990

20 Gln Ala Thr Lys Tyr Asn Ser Glu Thr Val Asp Lys Lys Ile Glu Glu  
 995 1000 1005

Phe Leu Ser Ser Phe Glu Glu Lys Ile Glu Asn Leu Thr Glu Asp  
 1010 1015 1020

25 Ala Phe Asn Thr Gln Val Thr Ala Leu Ile Lys Leu Lys Glu Cys  
 1025 1030 1035  
 Glu Asp Thr His Leu Gly Glu Glu Val Asp Arg Asn Trp Asn Glu  
 1040 1045 1050

30 Val Val Thr Gln Gln Tyr Leu Phe Asp Arg Leu Ala His Glu Ile  
 1055 1060 1065

35 Glu Ala Leu Lys Ser Phe Ser Lys Ser Asp Leu Val Ser Trp Phe  
 1070 1075 1080

40 Lys Ala His Arg Gly Pro Gly Ser Lys Met Leu Ser Val His Val  
 1085 1090 1095

45 Val Gly Tyr Gly Lys Tyr Glu Leu Glu Glu Asp Gly Ala Pro Val  
 1100 1105 1110

50 Cys Glu Asp Pro Asn Ser Arg Glu Gly Met Gln Leu Ile Tyr Leu  
 1115 1120 1125

Pro Pro Ser Pro Leu Leu Ala Glu Ser Thr Thr Pro Ile Thr Asp  
 1130 1135 1140

55 Ile Arg Ala Phe Thr Ala Thr Leu Ser Leu Phe Pro Tyr His Lys  
 1145 1150 1155



39/67

Ile Val Lys  
1160

5 <210> 11  
<211> 1019  
<212> PRT  
<213> Homo sapiens

10 <400> 11

Met Arg Tyr Arg Leu Ala Trp Leu Leu His Pro Ala Leu Pro Ser Thr  
1 5 10 15

15

Phe Arg Ser Val Leu Gly Ala Arg Leu Pro Pro Pro Glu Arg Leu Cys  
20 25 30

20 Gly Phe Gln Lys Lys Thr Tyr Ser Lys Met Asn Asn Pro Ala Ile Lys  
35 40 45

Arg Ile Gly Asn His Ile Thr Lys Ser Pro Glu Asp Lys Arg Glu Tyr  
25 50 55 60  
Arg Gly Leu Glu Leu Ala Asn Gly Ile Lys Val Leu Leu Met Ser Asp  
65 70 75 80

30 Pro Thr Thr Asp Lys Ser Ser Ala Ala Leu Asp Val His Ile Gly Ser  
85 90 95

35 Leu Ser Asp Pro Pro Asn Ile Ala Gly Leu Ser His Phe Cys Glu His  
100 105 110

Met Leu Phe Leu Gly Thr Lys Lys Tyr Pro Lys Glu Asn Glu Tyr Ser  
115 120 125

40

Gln Phe Leu Ser Glu His Ala Gly Ser Ser Asn Ala Phe Thr Ser Gly  
130 135 140

45

Glu His Thr Asn Tyr Tyr Phe Asp Val Ser His Glu His Leu Glu Gly  
145 150 155 160

50 Ala Leu Asp Arg Phe Ala Gln Phe Phe Leu Cys Pro Leu Phe Asp Glu  
165 170 175

Ser Cys Lys Asp Arg Glu Val Asn Ala Val Asp Ser Glu His Glu Lys  
180 185 190

Asn Val Met Asn Asp Ala Trp Arg Leu Phe Gln Leu Glu Lys Ala Thr  
195 200 205

40/67

Gly Asn Pro Lys His Pro Phe Ser Lys Phe Gly Thr Gly Asn Lys Tyr  
 210 215 220  
 5

Thr Leu Glu Thr Arg Pro Asn Gln Glu Gly Ile Asp Val Arg Gln Glu  
 225 230 235 240  
 10

Leu Leu Lys Phe His Ser Ala Tyr Tyr Ser Ser Asn Leu Met Ala Val  
 245 250 255  
 15

Cys Val Leu Gly Arg Glu Ser Leu Asp Asp Leu Thr Asn Leu Val Val  
 260 265 270  
 20

Lys Leu Phe Ser Glu Val Glu Asn Lys Asn Val Pro Leu Pro Glu Phe  
 275 280 285  
 25

Pro Glu His Pro Phe Gln Glu Glu His Leu Lys Gln Leu Tyr Lys Ile  
 290 295 300  
 Val Pro Ile Lys Asp Ile Arg Asn Leu Tyr Val Thr Phe Pro Ile Pro  
 305 310 315 320  
 30

Asp Leu Gln Lys Tyr Tyr Lys Ser Asn Pro Gly His Tyr Leu Gly His  
 325 330 335  
 35

Leu Ile Gly His Glu Gly Pro Gly Ser Leu Leu Ser Glu Leu Lys Ser  
 340 345 350  
 Lys Gly Trp Val Asn Thr Leu Val Gly Gly Gln Lys Glu Gly Ala Arg  
 355 360 365  
 40

Gly Phe Met Phe Phe Ile Ile Asn Val Asp Leu Thr Glu Glu Gly Leu  
 370 375 380  
 45

Leu His Val Glu Asp Ile Ile Leu His Met Phe Gln Tyr Ile Gln Lys  
 385 390 395 400  
 50

Leu Arg Ala Glu Gly Pro Gln Glu Trp Val Phe Gln Glu Cys Lys Asp  
 405 410 415  
 55

Leu Asn Ala Val Ala Phe Arg Phe Lys Asp Lys Glu Arg Pro Arg Gly  
 420 425 430  
 Tyr Thr Ser Lys Ile Ala Gly Ile Leu His Tyr Tyr Pro Leu Glu Glu  
 435 440 445

41/67

Val Leu Thr Ala Glu Tyr Leu Leu Glu Glu Phe Arg Pro Asp Leu Ile  
450 455 460

5  
Glu Met Val Leu Asp Lys Leu Arg Pro Glu Asn Val Arg Val Ala Ile  
465 470 475 480

10 Val Ser Lys Ser Phe Glu Gly Lys Thr Asp Arg Thr Glu Glu Trp Tyr  
485 490 495

15 Gly Thr Gln Tyr Lys Gln Glu Ala Ile Pro Asp Glu Val Ile Lys Lys  
500 505 510

20 Trp Gln Asn Ala Asp Leu Asn Gly Lys Phe Lys Leu Pro Thr Lys Asn  
515 520 525

Glu Phe Ile Pro Thr Asn Phe Glu Ile Leu Pro Leu Glu Lys Glu Ala  
530 535 540

25 Thr Pro Tyr Pro Ala Leu Ile Lys Asp Thr Val Met Ser Lys Leu Trp  
545 550 555 560

30 Phe Lys Gln Asp Asp Lys Lys Lys Lys Pro Lys Ala Cys Leu Asn Phe  
565 570 575

Glu Phe Phe Ser Pro Phe Ala Tyr Val Asp Pro Leu His Cys Asn Met  
580 585 590

35  
Ala Tyr Leu Tyr Leu Glu Leu Leu Lys Asp Ser Leu Asn Glu Tyr Ala  
595 600 605

40 Tyr Ala Ala Glu Leu Ala Gly Leu Ser Tyr Asp Leu Gln Asn Thr Ile  
610 615 620

45 Tyr Gly Met Tyr Leu Ser Val Lys Gly Tyr Asn Asp Lys Gln Pro Ile  
625 630 635 640

50 Leu Leu Lys Lys Ile Ile Glu Lys Met Ala Thr Phe Glu Ile Asp Glu  
645 650 655

Lys Arg Phe Glu Ile Ile Lys Glu Ala Tyr Met Arg Ser Leu Asn Asn  
660 665 670

55  
Phe Arg Ala Glu Gln Pro His Gln His Ala Met Tyr Tyr Leu Arg Leu  
675 680 685

42/67

Leu Met Thr Glu Val Ala Trp Thr Lys Asp Glu Leu Lys Glu Ala Leu  
 690 695 700

5 Asp Asp Val Thr Leu Pro Arg Leu Lys Ala Phe Ile Pro Gln Leu Leu  
 705 710 715 720

10 Ser Arg Leu His Ile Glu Ala Leu Leu His Gly Asn Ile Thr Lys Gln  
 725 730 735

15 Ala Ala Leu Gly Ile Met Gln Met Val Glu Asp Thr Leu Ile Glu His  
 740 745 750

Ala His Thr Lys Pro Leu Leu Pro Ser Gln Leu Val Arg Tyr Arg Glu  
 755 760 765

20 Val Gln Leu Pro Asp Arg Gly Trp Phe Val Tyr Gln Gln Arg Asn Glu  
 770 775 780

Val His Asn Asn Cys Gly Ile Glu Ile Tyr Tyr Gln Thr Asp Met Gln  
 785 790 795 800

25 Ser Thr Ser Glu Asn Met Phe Leu Glu Leu Phe Cys Gln Ile Ile Ser  
 805 810 815

30 Glu Pro Cys Phe Asn Thr Leu Arg Thr Lys Glu Gln Leu Gly Tyr Ile  
 820 825 830

35 Val Phe Ser Gly Pro Arg Arg Ala Asn Gly Ile Gln Ser Leu Arg Phe  
 835 840 845

40 Ile Ile Gln Ser Glu Lys Pro Pro His Tyr Leu Glu Ser Arg Val Glu  
 850 855 860

45 Ala Phe Leu Ile Thr Met Glu Lys Ser Ile Glu Asp Met Thr Glu Glu  
 865 870 875 880

Ala Phe Gln Lys His Ile Gln Ala Leu Ala Ile Arg Arg Leu Asp Lys  
 885 890 895

50 Pro Lys Lys Leu Ser Ala Glu Cys Ala Lys Tyr Trp Gly Glu Ile Ile  
 900 905 910

55 Ser Gln Gln Tyr Asn Phe Asp Arg Asp Asn Thr Glu Val Ala Tyr Leu  
 915 920 925

Lys Thr Leu Thr Lys Glu Asp Ile Ile Lys Phe Tyr Lys Glu Met Leu

43/67

930                                      935                                      940  
 Ala Val Asp Ala Pro Arg Arg His Lys Val Ser Val His Val Leu Ala  
 5 945                                      950                                      955                                      960  
 Arg Glu Met Asp Ser Cys Pro Val Val Gly Glu Phe Pro Cys Gln Asn  
 10                                      965                                      970                                      975  
 Asp Ile Asn Leu Ser Gln Ala Pro Ala Leu Pro Gln Pro Glu Val Ile  
 15                                      980                                      985                                      990  
 Gln Asn Met Thr Glu Phe Lys Arg Gly Leu Pro Leu Phe Pro Leu Val  
 20                                      995                                      1000                                      1005  
 Lys Pro His Ile Asn Phe Met Ala Ala Lys Leu  
 1010                                      1015  
 <210> 12  
 <211> 1265  
 <212> PRT  
 25 <213> Arabidopsis thaliana  
 <400> 12  
 Met Ala Ser Ser Ser Ser Ser Ile Phe Thr Gly Val Lys Phe Ser Pro  
 30 1                                      5                                      10                                      15  
 Ile Leu Ala Pro Phe Asn Ser Gly Asp Ser Arg Arg Ser Arg Tyr Leu  
 35                                      20                                      25                                      30  
 Lys Asp Ser Arg Asn Lys Val Arg Phe Asn Pro Ser Ser Pro Arg Leu  
 40                                      35                                      40                                      45  
 Thr Pro His Arg Val Arg Val Glu Ala Pro Ser Leu Ile Pro Tyr Asn  
 50                                      50                                      55                                      60  
 Gly Leu Trp Ala Ala Gln Pro Asn Ser His Lys Gly Arg Leu Lys Arg  
 45 65                                      70                                      75                                      80  
 Asn Ile Val Ser Gly Lys Glu Ala Thr Gly Ile Ser Leu Ser Gln Gly  
 50                                      85                                      90                                      95  
 Arg Asn Phe Cys Leu Thr Cys Lys Arg Asn Gln Ala Gly Ile Arg Arg  
 55                                      100                                      105                                      110  
 Ala Leu Pro Ser Ala Phe Val Asp Arg Thr Ala Phe Ser Leu Ser Arg  
 115                                      120                                      125

Ser Ser Leu Thr Ser Ser Leu Arg Lys His Ser Gln Ile Val Asn Ala  
 130 135 140  
 5  
 Thr Leu Gly Pro Asp Glu Pro His Ala Ala Gly Thr Ala Trp Pro Asp  
 145 150 155 160  
 10 Gly Ile Val Ala Glu Arg Gln Asp Leu Asp Leu Leu Pro Pro Glu Ile  
 165 170 175  
 15 Asp Ser Ala Glu Leu Glu Ala Phe Leu Gly Cys Glu Leu Pro Ser His  
 180 185 190  
 20 Pro Lys Leu His Arg Gly Gln Leu Lys Asn Gly Leu Arg Tyr Leu Ile  
 195 200 205  
 Leu Pro Asn Lys Val Pro Pro Asn Arg Phe Glu Ala His Met Glu Val  
 210 215 220  
 25 His Val Gly Ser Ile Asp Glu Glu Glu Asp Glu Gln Gly Ile Ala His  
 225 230 235 240  
 30 Met Ile Glu His Val Ala Phe Leu Gly Ser Lys Lys Arg Glu Lys Leu  
 245 250 255  
 35 Leu Gly Thr Gly Ala Arg Ser Asn Ala Tyr Thr Asp Phe His His Thr  
 260 265 270  
 Val Phe His Ile His Ser Pro Thr His Thr Lys Asp Ser Glu Asp Asp  
 275 280 285  
 40  
 Leu Phe Pro Ser Val Leu Asp Ala Leu Asn Glu Ile Ala Phe His Pro  
 290 295 300  
 45 Lys Phe Leu Ser Ser Arg Val Glu Lys Glu Arg Arg Ala Ile Leu Ser  
 305 310 315 320  
 50 Glu Leu Gln Met Met Asn Thr Ile Glu Tyr Arg Val Asp Cys Gln Leu  
 325 330 335  
 55 Leu Gln His Leu His Ser Glu Asn Lys Leu Gly Arg Arg Phe Pro Ile  
 340 345 350  
 Gly Leu Glu Glu Gln Ile Lys Lys Trp Asp Val Asp Lys Ile Arg Lys  
 355 360 365

45/67

Phe His Glu Arg Trp Tyr Phe Pro Ala Asn Ala Thr Leu Tyr Ile Val  
 370 375 380  
 5  
 Gly Asp Ile Asp Asn Ile Pro Arg Ile Val His Asn Ile Glu Ala Val  
 385 390 395 400  
 10 Phe Gly Lys Asn Gly Leu Asp Asn Glu Ser Thr Pro Ser Ser Pro Ser  
 405 410 415  
 15 Pro Gly Ala Phe Gly Ala Met Ala Asn Phe Leu Val Pro Lys Leu Pro  
 420 425 430  
 Ala Gly Leu Gly Gly Thr Phe Ser Asn Glu Lys Thr Asn Thr Ala Asp  
 435 440 445  
 20 Gln Ser Lys Met Ile Lys Arg Glu Arg His Ala Ile Arg Pro Pro Val  
 450 455 460  
 Glu His Asn Trp Ser Leu Pro Gly Thr Ser Val Asp Leu Lys Pro Pro  
 25 465 470 475 480  
 Gln Ile Phe Lys His Glu Leu Leu Gln Asn Phe Ala Ile Asn Met Phe  
 485 490 495  
 30  
 Cys Lys Ile Pro Val Ser Lys Val Gln Thr Phe Gly Asp Leu Arg Asn  
 500 505 510  
 35  
 Val Leu Met Lys Arg Ile Phe Leu Ser Ala Leu His Phe Arg Ile Asn  
 515 520 525  
 40 Thr Arg Tyr Lys Ser Ser Asn Pro Pro Phe Thr Ser Val Glu Leu Asp  
 530 535 540  
 His Ser Asp Ser Gly Arg Glu Gly Cys Thr Val Thr Thr Leu Thr Val  
 45 545 550 555 560  
 Thr Ala Glu Pro Gln Asn Trp Gln Asn Ala Val Lys Val Ala Val Gln  
 565 570 575  
 50  
 Glu Val Arg Arg Leu Lys Glu Phe Gly Val Thr Arg Gly Glu Leu Thr  
 580 585 590  
 55  
 Arg Tyr Met Asp Ala Leu Leu Lys Asp Ser Glu His Leu Ala Ala Met  
 595 600 605

46/67

Ile Asp Asn Val Ser Ser Val Asp Asn Leu Asp Phe Ile Met Glu Ser  
 610 615 620

5 Asp Ala Leu Ser His Thr Val Met Asp Gln Thr Gln Gly His Glu Thr  
 625 630 635 640

10 Leu Val Ala Val Ala Gly Thr Val Thr Leu Glu Glu Val Asn Thr Val  
 645 650 655

15 Gly Ala Lys Val Leu Glu Phe Ile Ser Asp Phe Gly Arg Pro Thr Ala  
 660 665 670

Leu Leu Pro Ala Ala Ile Val Ala Cys Val Pro Thr Lys Val His Val  
 675 680 685

20 Asp Gly Val Gly Glu Ser Asp Phe Asn Ile Ser Pro Asp Glu Ile Ile  
 690 695 700

25 Glu Ser Val Lys Ser Gly Leu Leu Ala Pro Ile Glu Ala Glu Pro Glu  
 705 710 715 720

Leu Glu Val Pro Lys Glu Leu Ile Ser Gln Ser Gln Leu Lys Glu Leu  
 725 730 735

30 Thr Leu Gln Arg Asn Pro Cys Phe Val Pro Ile Pro Gly Ser Gly Leu  
 740 745 750

35 Thr Lys Leu His Asp Lys Glu Thr Gly Ile Thr Gln Leu Arg Leu Ser  
 755 760 765

40 Asn Gly Ile Ala Val Asn Tyr Lys Lys Ser Thr Thr Glu Ser Arg Ala  
 770 775 780

45 Gly Val Met Arg Leu Ile Val Gly Gly Gly Arg Ala Ala Glu Thr Ser  
 785 790 795 800

Asp Ser Lys Gly Ala Val Val Val Gly Val Arg Thr Leu Ser Glu Gly  
 805 810 815

50 Gly Arg Val Gly Asp Phe Ser Arg Glu Gln Val Glu Leu Phe Cys Val  
 820 825 830

55 Asn His Leu Ile Asn Cys Ser Leu Glu Ser Thr Glu Glu Phe Ile Ala  
 835 840 845

Met Glu Phe Arg Phe Thr Leu Arg Asp Asn Gly Met Gln Ala Ala Phe



47/67

850

855

860

Gln Leu Leu His Met Val Leu Glu Arg Ser Val Trp Leu Glu Asp Ala  
 5 865 870 875 880

Phe Asp Arg Ala Arg Gln Leu Tyr Leu Ser Tyr Phe Arg Ser Ile Pro  
 10 885 890 895

Lys Ser Leu Glu Arg Ala Thr Ala His Lys Leu Met Ile Ala Met Leu  
 900 905 910

15

Asn Gly Asp Glu Arg Phe Val Glu Pro Thr Pro Lys Ser Leu Gln Ser  
 915 920 925  
 Leu Asn Leu Glu Ser Val Lys Asp Ala Val Met Ser His Phe Val Gly  
 930 935 940

20

Asp Asn Met Glu Val Ser Ile Val Gly Asp Phe Ser Glu Glu Glu Ile  
 945 950 955 960

25

Glu Arg Cys Ile Leu Asp Tyr Leu Gly Thr Val Lys Ala Ser His Asp  
 965 970 975

Ser Ala Lys Pro Pro Gly Ser Glu Pro Ile Leu Phe Arg Gln Pro Thr  
 980 985 990

Ala Gly Leu Gln Phe Gln Gln Val Phe Leu Lys Asp Thr Asp Glu Arg  
 35 995 1000 1005

Ala Cys Ala Tyr Ile Ala Gly Pro Ala Pro Asn Arg Trp Gly Phe  
 1010 1015 1020

40

Thr Val Asp Gly Asp Asp Leu Phe Gln Ser Val Ser Lys Leu Pro  
 1025 1030 1035

45

Val Ala His Asp Gly Leu Leu Lys Ser Glu Glu Gln Leu Leu Glu  
 1040 1045 1050

Gly Gly Asp Arg Glu Leu Gln Lys Lys Leu Arg Ala His Pro Leu  
 1055 1060 1065

Phe Phe Gly Val Thr Met Gly Leu Leu Ala Glu Ile Ile Asn Ser  
 55 1070 1075 1080

Arg Leu Phe Thr Thr Val Arg Asp Ser Leu Gly Leu Thr Tyr Asp  
 1085 1090 1095

5 Val Ser Phe Glu Leu Asn Leu Phe Asp Arg Leu Lys Leu Gly Trp  
 1100 1105 1110  
 Tyr Val Ile Ser Val Thr Ser Thr Pro Gly Lys Val Tyr Lys Ala  
 1115 1120 1125  
 10 Val Asp Ala Cys Lys Asn Val Leu Arg Gly Leu His Ser Asn Gln  
 1130 1135 1140  
 15 Ile Ala Pro Arg Glu Leu Asp Arg Ala Lys Arg Thr Leu Leu Met  
 1145 1150 1155  
 Arg His Glu Ala Glu Leu Lys Ser Asn Ala Tyr Trp Leu Asn Leu  
 1160 1165 1170  
 20 Leu Ala His Leu Gln Ala Ser Ser Val Gln Arg Lys Glu Leu Ser  
 1175 1180 1185  
 25 Cys Ile Lys Glu Leu Val Ser Leu Tyr Glu Ala Ala Ser Ile Glu  
 1190 1195 1200  
 30 Asp Ile Tyr Leu Ala Tyr Asn Gln Leu Arg Val Asp Glu Asp Ser  
 1205 1210 1215  
 35 Leu Tyr Ser Cys Ile Gly Ile Ala Gly Ala Gln Ala Gly Glu Glu  
 1220 1225 1230  
 Ile Thr Val Leu Ser Glu Glu Glu Glu Pro Glu Asp Val Phe Ser  
 1235 1240 1245  
 40 Gly Val Val Pro Val Gly Arg Gly Ser Ser Met Thr Thr Arg Pro  
 1250 1255 1260  
 45 Thr Thr  
 1265  
 50 <210> 13  
 <211> 534  
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 <213> Homo sapiens  
 <400> 13  
 55 Met Arg Pro Asp Asp Lys Tyr His Glu Lys Gln Ala Gln Val Glu Ala  
 1 5 10 15

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Thr Lys Leu Lys Gln Lys Val Glu Ala Leu Ser Pro Gly Asp Arg Gln  
 20 25 30

5 Gln Ile Tyr Glu Lys Gly Leu Glu Leu Arg Ser Gln Gln Ser Lys Pro  
 35 40 45

10 Gln Asp Ala Ser Cys Leu Pro Ala Leu Lys Val Ser Asp Ile Glu Pro  
 50 55 60

Thr Ile Pro Val Thr Glu Leu Asp Val Val Leu Thr Ala Gly Asp Ile  
 65 70 75 80

15 Pro Val Gln Tyr Cys Ala Gln Pro Thr Asn Gly Met Val Tyr Phe Arg  
 85 90 95

20 Ala Phe Ser Ser Leu Asn Thr Leu Pro Glu Glu Leu Arg Pro Tyr Val  
 100 105 110

25 Pro Leu Phe Cys Ser Val Leu Thr Lys Leu Gly Cys Gly Leu Leu Asp  
 115 120 125

Tyr Arg Glu Gln Ala Gln Gln Ile Glu Leu Lys Thr Gly Gly Met Ser  
 130 135 140

30 Ala Ser Pro His Val Leu Pro Asp Asp Ser His Met Asp Thr Tyr Glu  
 145 150 155 160

35 Gln Gly Val Leu Phe Ser Ser Leu Cys Leu Asp Arg Asn Leu Pro Asp  
 165 170 175

40 Met Met Gln Leu Trp Ser Glu Ile Phe Asn Asn Pro Cys Phe Glu Glu  
 180 185 190

45 Glu Glu His Phe Lys Val Leu Val Lys Met Thr Ala Gln Glu Leu Ala  
 195 200 205

Asn Gly Ile Pro Asp Ser Gly His Leu Tyr Ala Ser Ile Arg Ala Gly  
 210 215 220

50 Arg Thr Leu Thr Pro Ala Gly Asp Leu Gln Glu Thr Phe Ser Gly Met  
 225 230 235 240

55 Asp Gln Val Arg Leu Met Lys Arg Ile Ala Glu Met Thr Asp Ile Lys  
 245 250 255

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Pro Ile Leu Arg Lys Leu Pro Arg Ile Lys Lys His Leu Leu Asn Gly  
260 265 270

5 Asp Asn Met Arg Cys Ser Val Asn Ala Thr Pro Gln Gln Met Pro Gln  
275 280 285

10 Thr Glu Lys Ala Val Glu Asp Phe Leu Arg Ser Ile Gly Arg Ser Lys  
290 295 300

Lys Glu Arg Arg Pro Val Arg Pro His Thr Val Glu Lys Pro Val Pro  
305 310 315 320  
15 Ser Ser Ser Gly Gly Asp Ala His Val Pro His Gly Ser Gln Val Ile  
325 330 335

20 Arg Lys Leu Val Met Glu Pro Thr Phe Lys Pro Trp Gln Met Lys Thr  
340 345 350

His Phe Leu Met Pro Phe Pro Val Asn Tyr Val Gly Glu Cys Ile Arg  
355 360 365  
25

Thr Val Pro Tyr Thr Asp Pro Asp His Ala Ser Leu Lys Ile Leu Ala  
370 375 380  
30

Arg Leu Met Thr Ala Lys Phe Leu His Thr Glu Ile Arg Glu Lys Gly  
385 390 395 400

35 Gly Ala Tyr Gly Gly Gly Ala Lys Leu Ser His Asn Gly Ile Phe Thr  
405 410 415

40 Leu Tyr Ser Tyr Arg Asp Pro Asn Thr Ile Glu Thr Leu Gln Ser Phe  
420 425 430

Gly Lys Ala Val Asp Trp Ala Lys Ser Gly Lys Phe Thr Gln Gln Asp  
435 440 445  
45

Ile Asp Glu Ala Lys Leu Ser Val Phe Ser Thr Val Asp Ala Pro Val  
450 455 460  
50

Ala Pro Ser Asp Lys Gly Met Asp His Phe Leu Tyr Gly Leu Ser Asp  
465 470 475 480

55 Glu Met Lys Gln Ala His Arg Glu Gln Leu Phe Ala Val Ser His Asp  
485 490 495

Lys Leu Leu Ala Val Ser Asp Arg Tyr Leu Gly Thr Gly Lys Ser Thr

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500

505

510

5 His Gly Leu Ala Ile Leu Gly Pro Glu Asn Pro Lys Ile Ala Lys Asp  
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Pro Ser Trp Ile Ile Arg  
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<211> 409  
15 <212> PRT  
<213> Bacillus subtilis  
  
<400> 14

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25 Asn Asn Pro Thr Val Arg Ser Val Ala Ile Gly Val Trp Ile Gly Thr  
20 25 30

Gly Ser Arg His Glu Thr Pro Glu Ile Asn Gly Ile Ser His Phe Leu  
35 40 45  
30

Glu His Met Phe Phe Lys Gly Thr Ser Thr Lys Ser Ala Arg Glu Ile  
50 55 60

35 Ala Glu Ser Phe Asp Arg Ile Gly Gly Gln Val Asn Ala Phe Thr Ser  
65 70 75 80

40 Lys Glu Tyr Thr Cys Tyr Tyr Ala Lys Val Leu Asp Glu His Ala Asn  
85 90 95

45 Tyr Ala Leu Asp Val Leu Ala Asp Met Phe Phe His Ser Thr Phe Asp  
100 105 110

Glu Asn Glu Leu Lys Lys Glu Lys Asn Val Val Tyr Glu Glu Ile Lys  
115 120 125  
50

Met Tyr Glu Asp Ala Pro Asp Asp Ile Val His Asp Leu Leu Ser Lys  
130 135 140

55 Ala Thr Tyr Gly Asn His Ser Leu Gly Tyr Pro Ile Leu Gly Thr Glu  
145 150 155 160

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Glu Thr Leu Ala Ser Phe Asn Gly Asp Ser Leu Arg Gln Tyr Met His  
 165 170 175

5 Asp Tyr Tyr Thr Pro Asp Arg Val Val Ile Ser Val Ala Gly Asn Ile  
 180 185 190

10 Ser Asp Ser Phe Ile Lys Asp Val Glu Lys Trp Phe Gly Ser Tyr Glu  
 195 200 205

Ala Lys Gly Lys Ala Thr Gly Leu Glu Lys Pro Glu Phe His Thr Glu  
 210 215 220

15 Lys Leu Thr Arg Lys Lys Glu Thr Glu Gln Ala His Leu Cys Leu Gly  
 225 230 235 240

20 Phe Lys Gly Leu Glu Val Gly His Glu Arg Ile Tyr Asp Leu Ile Val  
 245 250 255

25 Leu Asn Asn Val Leu Gly Gly Ser Met Ser Ser Arg Leu Phe Gln Asp  
 260 265 270

Val Arg Glu Asp Lys Gly Leu Ala Tyr Ser Val Tyr Ser Tyr His Ser  
 275 280 285

30 Ser Tyr Glu Asp Ser Gly Met Leu Thr Ile Tyr Gly Gly Thr Gly Ala  
 290 295 300

35 Asn Gln Leu Gln Gln Leu Ser Glu Thr Ile Gln Glu Thr Leu Ala Thr  
 305 310 315 320

40 Leu Lys Arg Asp Gly Ile Thr Ser Lys Glu Leu Glu Asn Ser Lys Glu  
 325 330 335

45 Gln Met Lys Gly Ser Leu Met Leu Ser Leu Glu Ser Thr Asn Ser Lys  
 340 345 350

Met Ser Arg Asn Gly Lys Asn Glu Leu Leu Leu Gly Lys His Lys Thr  
 355 360 365

50 Leu Asp Glu Ile Ile Asn Glu Leu Asn Ala Val Asn Leu Glu Arg Val  
 370 375 380

55 Asn Gly Leu Ala Arg Gln Leu Phe Thr Glu Asp Tyr Ala Leu Ala Leu  
 385 390 395 400

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Ile Ser Pro Ser Gly Asn Met Pro Ser  
405

5 <210> 15  
<211> 438  
<212> PRT  
<213> Mycobacterium tuberculosis

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1 5 10 15

15 Arg Thr Thr Leu Pro Gly Gly Leu Arg Val Val Thr Glu Phe Leu Pro  
20 25 30

20 Ala Val His Ser Ala Ser Val Gly Val Trp Val Gly Val Gly Ser Arg  
35 40 45

25 Asp Glu Gly Ala Thr Val Ala Gly Ala Ala His Phe Leu Glu His Leu  
50 55 60

30 Leu Phe Lys Ser Thr Pro Thr Arg Ser Ala Val Asp Ile Ala Gln Ala  
65 70 75 80

35 Met Asp Ala Val Gly Gly Glu Leu Asn Ala Phe Thr Ala Lys Glu His  
85 90 95

40 Thr Cys Tyr Tyr Ala His Val Leu Gly Ser Asp Leu Pro Leu Ala Val  
100 105 110

45 Asp Leu Val Ala Asp Val Val Leu Asn Gly Arg Cys Ala Ala Asp Asp  
115 120 125

50 Val Glu Val Glu Arg Asp Val Val Leu Glu Glu Ile Ala Met Arg Asp  
130 135 140

55 Asp Asp Pro Glu Asp Ala Leu Ala Asp Met Phe Leu Ala Ala Leu Phe  
145 150 155 160

60 Gly Asp His Pro Val Gly Arg Pro Val Ile Gly Ser Ala Gln Ser Val  
165 170 175

65 Ser Val Met Thr Arg Ala Gln Leu Gln Ser Phe His Leu Arg Arg Tyr  
180 185 190

Thr Pro Glu Arg Met Val Val Ala Ala Ala Gly Asn Val Asp His Asp

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195

200

205

5 Gly Leu Val Ala Leu Val Arg Glu His Phe Gly Ser Arg Leu Val Arg  
 210 215 220

Gly Arg Arg Pro Val Ala Pro Arg Lys Gly Thr Gly Arg Val Asn Gly  
 225 230 235 240  
 10 Ser Pro Arg Leu Thr Leu Val Ser Arg Asp Ala Glu Gln Thr His Val  
 245 250 255

15 Ser Leu Gly Ile Arg Thr Pro Gly Arg Gly Trp Glu His Arg Trp Ala  
 260 265 270

Leu Ser Val Leu His Thr Ala Leu Gly Gly Gly Leu Ser Ser Arg Leu  
 275 280 285  
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Phe Gln Glu Val Arg Glu Thr Arg Gly Leu Ala Tyr Ser Val Tyr Ser  
 290 295 300

25 Ala Leu Asp Leu Phe Ala Asp Ser Gly Ala Leu Ser Val Tyr Ala Ala  
 305 310 315 320

30 Cys Leu Pro Glu Arg Phe Ala Asp Val Met Arg Val Thr Ala Asp Val  
 325 330 335

35 Leu Glu Ser Val Ala Arg Asp Gly Ile Thr Glu Ala Glu Cys Gly Ile  
 340 345 350

Ala Lys Gly Ser Leu Arg Gly Gly Leu Val Leu Gly Leu Glu Asp Ser  
 355 360 365  
 40

Ser Ser Arg Met Ser Arg Leu Gly Arg Ser Glu Leu Asn Tyr Gly Lys  
 370 375 380

45 His Arg Ser Ile Glu His Thr Leu Arg Gln Ile Glu Gln Val Thr Val  
 385 390 395 400

50 Glu Glu Val Asn Ala Val Ala Arg His Leu Leu Ser Arg Arg Tyr Gly  
 405 410 415

55 Ala Ala Val Leu Gly Pro His Gly Ser Lys Arg Ser Leu Pro Gln Gln  
 420 425 430

Leu Arg Ala Met Val Gly  
 435



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<210> 16  
<211> 34  
5 <212> DNA  
<213> Artificial Sequence

<220>  
10 <223> Oligonucleotide

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aatagaagct tgtcgactga tctatccaaa actg 34

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<210> 17  
<211> 66  
<212> DNA  
<213> Artificial Sequence

20  
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<223> Oligonucleotide

<400> 17  
25 aaaagagctc ggccagatct tctagaggat ccaagaattc tgttttatat ttgttgtaaa 60  
aagtag 66

30 <210> 18  
<211> 37  
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<220>  
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40 ttttgaattc caagatctcc catgtctcta ctggttg 37

<210> 19  
<211> 41  
45 <212> DNA  
<213> Artificial Sequence

<220>  
50 <223> Oligonucleotide

<400> 19  
ccccgagctc gtcgaccctt ctcgaaagct ttaacgaacg c 41

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<210> 20  
<211> 43  
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<213> Artificial Sequence

<220>  
<223> Oligonucleotide

5 <400> 20  
ttttgaattc aaagaatgag atttccttca atttttactg cag 43

<210> 21  
<211> 37  
10 <212> DNA  
<213> Artificial Sequence

<220>  
<223> Oligonucleotide

15 <400> 21  
tttttctaga ctaggagggg tactcatact cctcggc 37

<210> 22  
20 <211> 33  
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<213> Artificial Sequence

<220>  
25 <223> Oligonucleotide

<400> 22  
cgaatgtcca tcgttgcgaa cctgcagaac ctg 33

30 <210> 23  
<211> 33  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Oligonucleotide

<400> 23  
caggttctgc aggttcctaa cgatggacat tcg 33

40 <210> 24  
<211> 33  
<212> DNA  
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45 <220>  
<223> Oligonucleotide

<400> 24  
50 cgaatgtcca tcgttaggaa cctgcagaac ctg 33

<210> 25  
<211> 33  
55 <212> DNA  
<213> Artificial Sequence

<220>

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<223> Oligonucleotide

<400> 25  
caggttctgc aggttcctaa cgatggacat tcg 33

5 <210> 26  
<211> 18  
<212> DNA  
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10 <220>  
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<400> 26  
tcgcagagaa cggatggc 18

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<210> 27  
<211> 36  
<212> DNA  
20 <213> Artificial Sequence

<220>  
<223> Oligonucleotide

25 <400> 27  
ttttgggcc ttcattgtga tacggtatct cttggc 36

<210> 28  
30 <211> 37  
<212> DNA  
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<220>  
35 <223> Oligonucleotide

<400> 28  
ttttctcgag aaggtggaac atactgccct gggatgg 37

40  
<210> 29  
<211> 38  
<212> DNA  
<213> Artificial Sequence

45  
<220>  
<223> Oligonucleotide

<400> 29  
50 ttttgagctc gtttaggaaa cgtccttggc ggagatgc 38

<210> 30  
<211> 40  
55 <212> DNA  
<213> Artificial Sequence

<220>  
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<400> 30  
tttttctaga cactgcgaat ccatggtata aaccaaaacc 40  
<210> 31  
5 <211> 24  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
10 <223> Oligonucleotide  
  
<400> 31  
gtcgttggtc atggacatac ctcc 24  
  
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<210> 32  
<211> 26  
<212> DNA  
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<223> Oligonucleotide  
  
<400> 32  
25 taaaaatggt cttctgcat ttctgg 26  
  
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<211> 32  
30 <212> DNA  
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<400> 33  
ggttcatatg cgccggagct cctcgacagc ag 32  
  
40 <210> 34  
<211> 37  
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<213> Artificial Sequence  
  
45 <220>  
<223> Oligonucleotide  
  
<400> 34  
50 ggttcctagg atccgcaagt ttgattccat tgcggtg 37  
  
<210> 35  
<211> 70  
<212> DNA  
55 <213> Artificial Sequence  
  
<220>  
<223> Oligonucleotide

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<400> 35  
ttaaagagta ccttggtat agaataccgt agagataaag acctgaatag agattgtact 60  
gagagtgcac 70

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<210> 36  
<211> 70  
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<213> Artificial Sequence

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<223> Oligonucleotide

<400> 36  
15 aggtattata actatattttc tgtatattttt atatattttt atttgccaag ctgtgcggtta 60  
tttcacaccg 70

20 <210> 37  
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25 <220>  
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30 ctttgggttaa agagtacctt ggc 23

<210> 38  
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35 <213> Artificial Sequence

<220>  
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40 <400> 38  
tactacgaaa agcgtgtgcg agg 23

<210> 39  
45 <211> 71  
<212> DNA  
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<220>  
50 <223> Oligonucleotide

<400> 39  
tagaaggcta ctcaaaagaa taaagttact ataaaatata ctgcggtata tagattgtac 60

55 tgagagtgcac c 71

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15 <210> 41  
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 <212> DNA  
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 20 <220>  
 <223> Oligonucleotide  
 <400> 41  
 25 cggaggggct ctatgataaa gg 22

<210> 42  
 <211> 23  
 <212> DNA  
 30 <213> Artificial Sequence  
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 <223> Oligonucleotide  
 35 <400> 42  
 gagtaactag ggcttctctt ccc 23

<210> 43  
 40 <211> 85  
 <212> PRT  
 <213> Homo sapiens  
 <400> 43  
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Ser Gly Leu Gln Arg Ala Glu Glu Ala Pro Arg Arg Gln Leu Arg Val  
 1 5 10 15

50 Ser Gln Arg Thr Asp Gly Glu Ser Arg Ala His Leu Gly Ala Leu Leu  
 20 25 30

Ala Arg Tyr Ile Gln Gln Ala Arg Lys Ala Pro Ser Gly Arg Met Ser  
 55 35 40 45

Ile Val Lys Asn Leu Gln Asn Leu Asp Pro Ser His Arg Ile Ser Asp  
 50 55 60

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Arg Asp Tyr Met Gly Trp Met Asp Phe Gly Arg Arg Ser Ala Glu Glu  
 65 70 75 80  
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 Tyr Glu Tyr Pro Ser  
 85  
 10  
 <210> 44  
 <211> 22  
 <212> PRT  
 <213> Homo sapiens  
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 Gln Leu Arg Val Ser Gln Arg Thr Asp Gly Glu Ser Arg Ala His Leu  
 20 1 5 10 15  
 Gly Ala Leu Leu Ala Arg  
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 25  
 <210> 45  
 <211> 19  
 <212> PRT  
 30 <213> Homo sapiens  
 <400> 45  
 35 Val Ser Gln Arg Thr Asp Gly Glu Ser Arg Ala His Leu Gly Ala Leu  
 1 5 10 15  
 Leu Ala Arg  
 40  
 <210> 46  
 <211> 51  
 45 <212> PRT  
 <213> Homo sapiens  
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 Tyr Ile Gln Gln Ala Arg Lys Ala Pro Ser Gly Arg Met Ser Ile Val  
 1 5 10 15  
 55 Lys Asn Leu Gln Asn Leu Asp Pro Ser His Arg Ile Ser Asp Arg Asp  
 20 25 30  
 Tyr Met Gly Trp Met Asp Phe Gly Arg Arg Ser Ala Glu Glu Tyr Glu  
 35 40 45

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Tyr Pro Ser  
50

5 <210> 47  
<211> 17  
<212> PRT  
<213> Homo sapiens

10  
<400> 47

Tyr Ile Gln Gln Ala Arg Lys Ala Pro Ser Gly Arg Met Ser Ile Val  
1 5 10 15

Lys

20 <210> 48  
<211> 16  
<212> PRT  
<213> Homo sapiens

25 <400> 48

Tyr Ile Gln Gln Ala Arg Lys Ala Pro Ser Gly Arg Met Ser Ile Val  
1 5 10 15

30 <210> 49  
<211> 13  
<212> PRT  
<213> Homo sapiens

35  
<400> 49

Asn Leu Gln Asn Leu Asp Pro Ser His Arg Ile Ser Asp  
1 5 10

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<210> 50  
<211> 23  
<212> PRT  
45 <213> Homo sapiens

<400> 50

50 Asn Leu Gln Asn Leu Asp Pro Ser His Arg Ile Ser Asp Arg Asp Tyr  
1 5 10 15

Met Gly Trp Met Asp Phe Gly  
55 20  
<210> 51  
<211> 34  
<212> PRT  
<213> Homo sapiens



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&lt;400&gt; 51

Asn Leu Gln Asn Leu Asp Pro Ser His Arg Ile Ser Asp Arg Asp Tyr  
5 1 5 10 15

Met Gly Trp Met Asp Phe Gly Arg Arg Ser Ala Glu Glu Tyr Glu Tyr  
20 25 30  
10

Pro Ser

15

&lt;210&gt; 53

&lt;211&gt; -12-

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

20

&lt;220&gt;

&lt;223&gt; Portion of fusion protein

&lt;400&gt; 53

25

Lys Arg Glu Ala Glu Ala Ser Gly Leu Gln Arg Ala  
1 5 10

30 &lt;210&gt; 54

&lt;211&gt; 9

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

35

&lt;400&gt; 54

Arg Met Ser Ile Val Lys Asn Leu Gln  
1 5

40

&lt;210&gt; 55

&lt;211&gt; 13

&lt;212&gt; PRT

45 &lt;213&gt; Homo sapiens

&lt;400&gt; 55

Asp Arg Asp Tyr Met Gly Trp Met Asp Phe Gly Arg Arg  
50 1 5 10

&lt;210&gt; 56

&lt;211&gt; 48

55 &lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Oligonucleotide MFalBNP (S)

<400> 56  
5 ggataaaaga gaggctgaag ctcacccgct gggcagcccc gggttcagc 48  
<210> 57  
<211> 48  
<212> DNA  
<213> Artificial Sequence  
10 <220>  
<223> MF1aBNP (AS)  
  
<400> 57  
15 gctgaaccgg gggtgcccag cgggtgagct tcagcctctc ttttatcc 48  
<210> 58  
<211> 34  
<212> DNA  
20 <213> Artificial Sequence  
<220>  
<223> Oligonucleotide BNP5'EcoRI  
  
25 <400> 58  
ttttgaattc atggatcccc agacagcacc ttcc 34  
<210> 59  
30 <211> 33  
<212> DNA  
<213> Artificial Sequence  
<220>  
35 <223> Oligonucleotide BNP3'XbaI  
  
<400> 59  
40 tttttctaga ttaatgccgc ctcagcactt tgc 33  
<210> 60  
<211> 48  
<212> DNA  
<213> Artificial Sequence  
45 <220>  
<223> Oligonucleotide MF1BNP (S)  
  
<400> 60  
50 ggataaaaga gaggctgaag ctcacccgct gggcagcccc gggttcagc 48  
<210> 61  
<211> 48  
55 <212> DNA  
<213> Artificial Sequence  
<220>  
<223> Oligonucleotide MF1BNP (AS)

<400> 61  
5 gctgaaccgg ggctgcccag cgggtgagct tcagcctctc ttttatcc 48  
<210> 62  
<211> 69  
<212> DNA  
<213> Artificial Sequence  
10 <220>  
<223> Oligonucleotide YPS15'GD400  
<400> 62  
15 aaaaagataa ggtgaacacc aagcatatag tataatatta cctaccacat gattgtactg 60  
agagtgcac 69  
<210> 63  
<211> 70  
20 <212> DNA  
<213> Artificial Sequence  
<220>  
<223> Oligonucleotide YPS13'GD400  
25 <400> 63  
aactccaact ggcttggaga tgtgaatgtc taaactttgt gcaacggttt ctgtgcggtg 60  
tttcacaccg 70  
30 <210> 64  
<211> 20  
<212> DNA  
<213> Artificial Sequence  
35 <220>  
<223> Oligonucleotide YPS15'DC  
<400> 64  
40 tcgtttcact gatgtgtccg 20  
<210> 65  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
45 <220>  
<223> Oligonucleotide YPS15'DC  
<400> 65  
50 gattataggc catatcccag g 21  
<210> 66  
<211> 13  
<212> PRT  
55 <213> Artificial Sequence  
<220>  
<223> Pitrilysin consensus sequence

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<221> VARIANT  
 <222> (1)...(13)  
 <223> Xaa = Any Amino Acid  
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 <221> VARIANT  
 <222> (1)...(13)  
 <223> Xaa = Any Amino Acid  
 10 <221> VARIANT  
 <222> (1)...(13)  
 <223> Xaa = Any Amino Acid  
 <221> UNSURE  
 15 <222> (0)...(0)  
 <400> 11  
 Gly Xaa Xaa His Xaa Xaa Glu His Xaa Xaa Xaa Xaa Gly  
 1 5 10  
 20  
 <210> 67  
 <211> 44  
 <212> PRT  
 25 <213> Artificial Sequence  
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 <223> Pitrilysin consensus sequence  
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 <223> Xaa = Any Amino Acid  
 35 <221> VARIANT  
 <222> (1)...(44)  
 <223> Xaa = Any Amino Acid  
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 40 <222> (1)...(44)  
 <223> Xaa = Any Amino Acid  
 <221> VARIANT  
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 45 <223> Xaa = any amino acid or absent  
 <221> VARIANT  
 <222> (35)...(35)  
 <223> Xaa = any amino acid or absent  
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 <400> 12  
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 1 5 10 15  
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
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67/67

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20 25 30  
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35 40